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Introduction to

IMMUNOCHEMICAL SPECIFICITY

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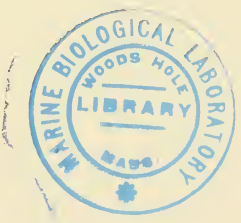
Introduction to

Immunochemical Specificity

by WILLIAM C. BOYD
Professor of Immunochemistry
Boston University School of Medicine



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Preface

This little book is intended to introduce the reader to our present knowledge of immunochemical specificity (a somewhat broader topic than the specificity of antibodies and antigens) by a discussion of some of the important modern advances in this field and an introductory account of certain earlier work that has served as a foundation for recent progress. Aimed at the nonspecialist as well as the specialist, the treatment is on the whole more elementary than that of my *Fundamentals of Immunology,* but in some ways more detailed and up to date.

The material is based primarily on a series of lectures which I had the privilege of giving in Moscow in the autumn of 1959. The book is not, however, a mere retranslation of the Russian text, but a thorough revision of the original English, with considerable additions.

Certain traces of the lecture form in which the material was originally cast still remain. Some of these may be disadvantages, but some of them perhaps may not be. The style of lectures can and, in my opinion, should be somewhat more informal than that of a textbook. The lecturer is also more or less expected to do certain things (and not to do others). He is expected, for example, to bring his audience up to date, even to the point of presenting some material not yet to be found in the textbooks and in some cases not yet published. He is expected, or at least allowed, to discuss certain aspects of his own work in more detail than might seem proper elsewhere. At the same time he is not required to cover the field

exhaustively and may be forgiven if, instead, he elects to emphasize those portions which are of special interest to him personally. He is also generally excused from presenting an exhaustive bibliography.

Most important of all, perhaps, lecturers are allowed to illustrate their talks with numerous lantern slides, a feature which often greatly increases the intelligibility of their presentation. This privilege is reflected in the present case by a relatively high proportion of figures and tables, which I hope will help in a similar way. In any case, the illustrations form an integral part of the plan of the book.

Although the topic is a specialized one, little previous knowledge of it is assumed on the part of the reader. An elementary knowledge of organic chemistry and, for the last two chapters, a slight acquaintance with the notation of partial differentiation should be sufficient.

The author is grateful to the John Simon Guggenheim Foundation for a fellowship that made the completion of this book possible, and to friends and colleagues who read and criticized portions of the manuscript.

Casa Rosada W. C. B.
May 1961

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CHAPTER 1

Antibodies I

Immunity

Basically speaking, immunity is the increased resistance to an infectious disease which often follows recovery from an initial attack. The degree of this immunity is different with different diseases and different patients and persists for varying periods of time. Recovery from certain virus diseases, such as yellow fever, is followed by a very high degree of resistance which seems to last for life in many patients. Recovery from the common cold, on the other hand, is followed by a very brief state of increased resistance, if indeed by any at all. We shall also apply the term immunity to the artificially increased resistance produced in a patient by injection or oral administration of living virus or living microorganisms or by injection of attenuated or dead virus or microorganisms or of antigenic products derived from such material. A patient whose resistance has been heightened by such treatment is said to have been immunized, although he may not be immune in the absolute sense of the word. Animals which have been caused to produce antibodies by such administration of antigen are also said to have been immunized, even though they may not have obtained increased resistance to any disease as a result.

Role of Antibodies in Immunity

The circulation of the immune animal often contains soluble protective proteins called antibodies, a term which is also applied to specifically reactive proteins produced in response to any antigen, whether it is derived from a pathogenic microorganism or not.

There is abundant evidence that antibodies play an important role in an individual's resistance to many diseases. For example, the transfer of antibody-containing blood from a convalescent patient to a susceptible person will often make the recipient temporarily immune to the disease from which the donor has just recovered (passive immunization). Transfer of antibody from an artificially immunized animal may be similarly effective. A decisive change in the sick patient's condition for the better ("crisis") many times coincides with the appearance of specific antibodies in the blood. The blood level of specific antibodies is often a fairly reliable index of the degree of a person's immunity.

It is a characteristic feature of antibodies that they react with the antigen which caused their production; in fact, new proteins appearing in the circulation which do not react in a detectable way with the antigen responsible for their production in general are not called antibodies. In a few instances antibodies have been observed which reacted with an antigen different from the one which caused them to be produced and did not react *visibly* with their own antigen (Hooker and Boyd, 1933; Clutton, Harington, and Yuill, 1938), but these are exceptions.

The reaction of antibodies with their antigen can have one or more of a number of effects: (a) Antibodies to toxins may neutralize the toxity of the antigen, and antibodies to viruses may neutralize the infectivity of the antigen. (b) Antibodies to soluble proteins and other soluble antigens may precipitate their antigen (Fig. 1-1). (c) Antibodies to microorganisms and foreign erythrocytes may cause the antigenic cells to stick together (agglutinate) (Fig. 1-2). (d) Antibodies to erythrocytes and certain microorganisms may cause the antigenic cells to disintegrate. This phenomenon is called lysis, and for its production the cooperation of certain normal components of plasma, collectively called complement, is required. (e) Antibodies to certain microorganisms, aided by complement, may cause the death of the antigenic cells (bactericidal effect). (f) Antibody to certain microorganisms causes the capsules of the microorganisms to swell visibly. This phenomenon is generally referred to by its German name *Quellung*. (g) Combination of antibody with microorganisms and other foreign cells generally makes the invaders more attractive to the leukocytes of the patient's circulation and thus pro-

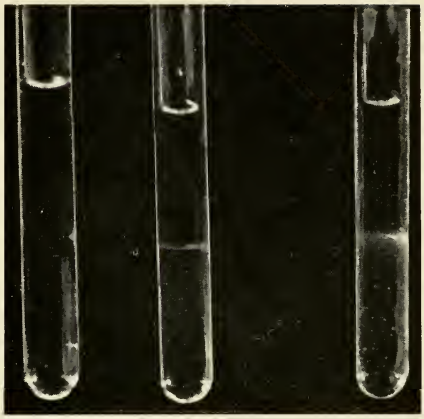

Fig. 1-1. Photographs of the precipitin reaction. Reading from left to right: negative reaction, weak positive reaction, strong positive reaction. These tests were carried out by the interfacial technique—placing a layer of diluted antigen over a layer of immune serum in a test tube).

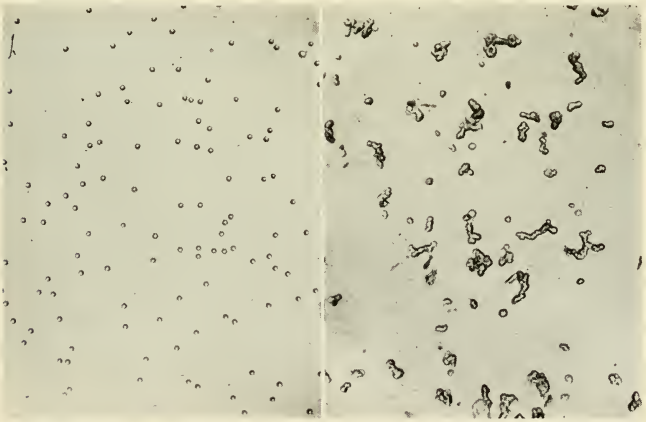

Fig. 1-2. Photomicrographs of unagglutinated red blood cells (left) and agglutinated cells (right).

motes phagocytosis. (h) Complement, if present, is generally taken up when antibody and antigen combine; thus, the occurrence of an otherwise undetectable antibody-antigen reaction can sometimes be deduced from observation of complement fixation alone. The classical Wassermann test for syphilis is based on this phenomenon. (i) The combination of antibody and antigen may lead to the release of histamine and other toxic substances from the tissues of the host, as in anaphylaxis and allergy.

All these effects of antibody, except probably those of class (i), are thought to be beneficial to the host and to aid in resistance to infection. All of them may, under suitable conditions, be utilized in laboratory studies.

However, although there is no doubt of the importance of antibodies in immunity, they are by no means the whole story, and the natural, more or less nonspecific mechanisms of resistance, such as impermeability of the skin and mucus membranes, and bactericidal power of these body surfaces, the rise in body temperature which often accompanies infection, the action of normal plasma components such as complement and properdin, and the ingestion of invading microorganisms by the leukocytes (phagocytosis), are also important. Indeed, of all the mechanisms of resistance, phagocytosis is probably by far the most important. However, we shall here be concerned with specific mechanisms of immunity and shall not further discuss these other tools of resistance.

Nature of Antibodies

In view of the importance of antibodies in immunity and of their theoretical interest as prime examples of specifically reacting biological substances, it is not surprising that many attempts have been made to study their chemical nature. Thus far it has not been possible to ascertain by direct chemical analysis the structural basis for the combining power and specificity of antibodies, because (a) it is not easy to obtain large amounts of purified antibodies and (b) protein chemistry is not far enough advanced for detailed knowledge of the structure of any antibody molecule to be obtained.

In spite of the difficulties, some preparations of purified antibody have been studied. There has also been analytical work on antibody-antigen compounds, which are more readily available in a relatively

pure state. Studies of the changes in the composition of blood following immunization have suggested that antibodies belong to the class of serum proteins called globulins. The distinction between serum albumin and serum globulins was originally based on solubility characteristics in neutral salt solutions (Cohn et al., 1940; Svensson, 1941). It is now based more on the observation that in an electric field the serum globulins move more slowly, at alkaline pH (Tiselius, 1937). In his classical paper, Tiselius (1937) pointed out that normal serum globulin showed components of at least three different electrophoretic mobilities, and he designated them as alpha, beta, and gamma globulins in order of decreasing mobility. Antibodies, with some possible exceptions, belong to the gamma globulin class. It is this group of globulins that increases most following immunization (Tiselius and Kabat, 1939).

In man, the rabbit, and many other species, antibodies are found by ultracentrifugal measurements to have the molecular weight characteristic of serum globulins, namely about 160,000. In the horse, pig,

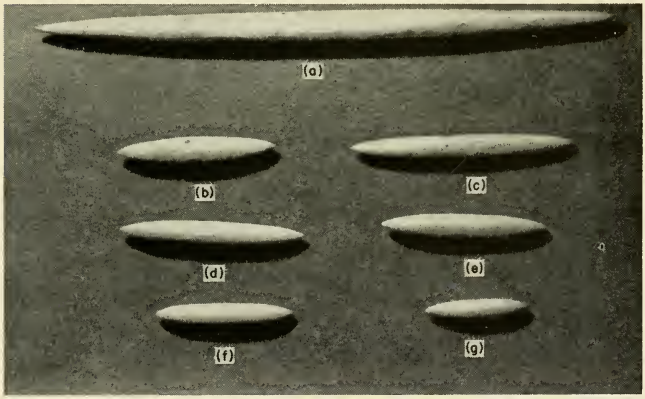

Fig. 1-3. Models of typical antibody molecules with human serum albumin for comparison. (a) Horse anti-pneumococcus antibody; (b) horse antitoxin; (c) rabbit anti-ovalbumin antibody; (d) human anti-pneumococcus antibody; (e) rabbit anti-pneumococcus antibody; (f) human gamma globulin; and (g) human serum albumin.

cow, and perhaps other species, anti-pneumococcus antibodies, for example, have a molecular weight of about 900,000. Certain antibodies in human blood seem to have molecular weights somewhere between these values (discussion in Boyd, 1956). From ultracentrifugal sedimentation constants, diffusion constants, and Perrin's (1936) relation between the "frictional ratio" and axial ratio of a prolate spheroid, the shape of protein molecules may be calculated. Photographs of models of typical antibody molecules, with human serum albumin for comparison, are shown in Fig. 1-3.

Actual photographs of antibody molecules, taken with the electron microscope, reveal, as far as the still inadequate resolving power of this instrument allows, a striking similarity to the models shown in Fig. 1-3 (see Fig. 1-4). Also of interest in the photograph is the apparent heterogeneity in size.

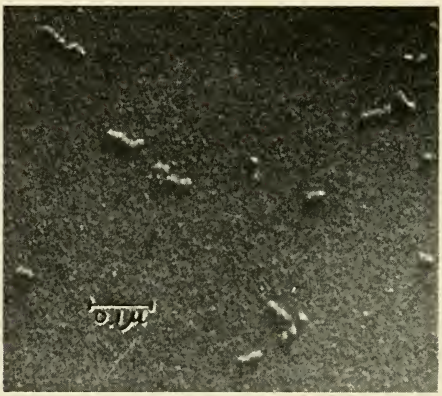

Fig. 1-4. Electron micrographs of rabbit antibody molecules. (Photograph by Dr. C. E. Hall.)

Specificity

It is a very old observation that immunity is specific. A child who has recovered from whooping cough is very unlikely to get this disease again in the immediate future, but his resistance to measles is not

any greater than before. Even recovery from the superficially very similar disease German measles does not seem to confer any immunity to ordinary measles, and the child generally catches all the common childhood infections, one after the other. If he misses one or more of them he remains susceptible to it, as he demonstrates by promptly coming down with it when exposed later in life, possibly from one of his own children. However, this specificity is not absolute. As an example we may mention that recovery from the relatively mild flea-borne typhus caused by *Rickettsia mooseri* is followed by immunity to the much more serious louse-borne typhus caused by *Rickettsia prowazeki* (Rivers, 1952).

Specificity of Antibodies

Just as the immunity following recovery from infections is relatively specific, so is the power of antibodies to react with antigens. Diphtheria antitoxin will neutralize diphtheria toxin and possibly save the life of a patient with diphtheria; it does not neutralize tetanus toxin and is of no value in the prevention or treatment of tetanus. In general, antibodies seem to be adapted to react just with the antigen which called forth their production (homologous antigen). But the specificity of antibodies, like the specificity of immunity, is not absolute. Antibodies produced by injecting rabbits with purified ovalbumin from the hen react also with the ovalbumin of various other birds such as the duck. A reaction of an antibody with a related antigen is called a cross-reaction. It is generally not as strong as the reaction of the antibody with the homologous antigen.

Today there is no doubt that the specificity of antibodies depends on their chemical structure. But there is as yet no agreement whether the specificity is a result of differences in amino acid composition or in amino acid sequence, or merely of the way in which the polypeptide chain is folded to produce the globular molecules shown in Fig. 1-3. Pauling (1940) proposed the latter view. It is a fact that amino acid analyses of antibodies have not yet revealed any clear-cut differences in amino acid composition between different antibodies or between particular antibodies and normal globulin (Boyd, 1956; Smith et al., 1955). If the differences were mainly in amino acid sequence and were confined to a "central, differential segment"

(Lederberg, 1959), they might be too minor to be found by the presently available methods of analysis.

Although the brilliant researches of Sanger (1956) into the sequence of amino acids in the polypeptide chain have resulted in the complete elucidation of the structure of insulin and a few other polypeptides, such analysis has not yet been carried very far with antibodies. However, we do know that the N-terminal sequence of all rabbit globulins thus far studied, including various antibodies, seems to be (Porter, 1950; McFadden and Smith, 1955):

Alanine—leucine—valine—aspartic acid—glutamic acid—

In contrast to the uniformity of rabbit globulins, horse globulins, whether antibody or normal globulin, have proved to be quite heterogeneous in this respect, all preparations exhibiting a wide variety of N-terminal groups (McFadden and Smith, 1955b). The globulins of man likewise differ among themselves in amino acid composition (Smith et al., 1955b; Putnam, 1955).

Powers of Discrimination of Antibodies

It was long ago suspected that the cross-reactions of antibodies with related antigens were due to chemical similarities between the homologous and the cross-reacting antigen. But in the absence of detailed information about the chemical structure of natural antigens (a situation which has improved only slightly since the earliest days of immunochemical work), it was not possible to state how great the chemical similarity between two antigens had to be to make cross-reaction possible or, to put it another way, how small a chemical differences antibodies could detect. Karl Landsteiner (1945) largely overcame this difficulty by the use of conjugated antigens.

It was known that chemical treatment (nitration, iodination, etc.) of protein antigens often changed the immunochemical specificity. Landsteiner showed that if simple chemical compounds were coupled chemically to protein antigens it was possible to produce antibodies which reacted specificially with the simple free compound (which Landsteiner called a hapten). Thus it was possible to observe serological reactions which depended only on the hapten, the structure of which was known, and not on the natural protein antigen of yet undetermined chemical structure.

As an example, let us take the aromatic amine, metanilic acid, and diazotize the amino group by treating the compound with nitrous acid (Fig. 1-5).

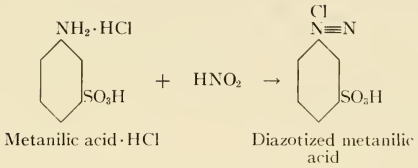

Fig. 1-5. Diazotization of metanilic acid.

The resulting diazonium salt will couple with phenols in alkaline solution to give colored azo dyes (Fig. 1-6). It will also couple with the phenolic group of the amino acid tyrosine, a constituent of most proteins.

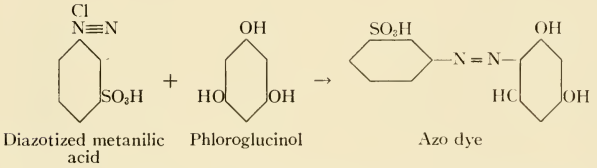

Fig. 1-6. Coupling of diazotized metanilic acid with phloroglucinol to form an azo dye.

Let us suppose we couple the diazotized metanilic acid (our hapten) with the mixture of proteins provided by horse serum. If we represent the horse serum proteins by H and the diazotized metanilic acid by M, we may reprsent the coupled azoprotein as HM. Injection of this compound HM into rabbits will usually cause the production of a number of different antibodies to the proteins of horse serum, which we may designate collectively as anti-H, and antibodies to metanilic acid, which we may designate as anti-M.

If, on testing the rabbit antibodies with the antigen HM we injected, we get a positive serological reaction (formation of a specific precipitate), we shall not know whether this is due to the union of

M and anti-M or the union of H and anti-H, or both. But let us suppose we couple diazotized metanilic acid with the proteins of chicken serum, which we may designate as C. Rabbit antibodies to horse serum proteins do not precipitate with chicken serum proteins, so the confusion caused by interference of the protein carrier is eliminated. If we mix our immune rabbit serum with CM and obtain a precipitate, we know it is due to the reaction of the anti-M of the rabbit serum with the M (metanilic acid) we have coupled with the chicken serum C (Fig. 1-7).

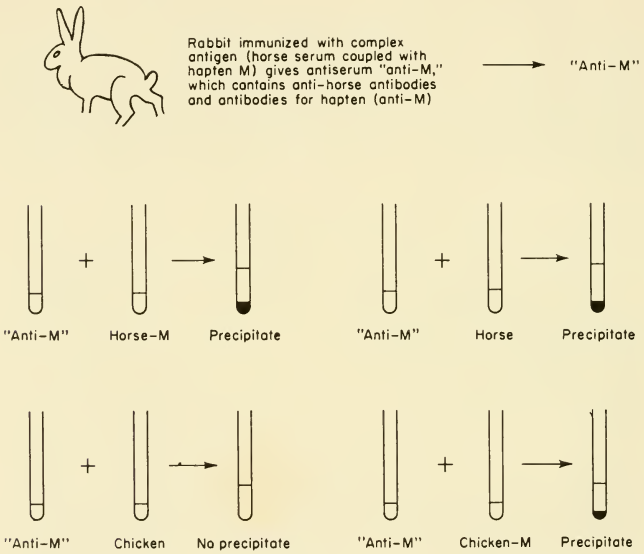

Fig. 1-7. Principle of detecting antibodies to a hapten independently of antibodies to the protein carrier.

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CHAPTER 2

Antibodies II

Specificity and Chemical Structure of Antigen

We are now in a position to investigate the effect on serological specificity of known variations, large or small, in the chemical composition of cross-reactive antigens. Suppose, for example, we replace the metanilic acid which we employed in making the azo chicken serum CM with a different aromatic amine, which we may designate as M′. Then if we test CM′ with our antiserum against horse-metanilic acid, a positive reaction will depend on whether M′ is sufficiently similar to M to combine with anti-M. If M′ is *m*-aminobenzoic acid or *m*-aminoarsenic acid, for example, a positive reaction may be obtained with an antiserum to horse-metanilic acid, although the amount of precipitate will be less. This shows that the antibodies to metanilic acid, though best adapted to the homologous hapten, can react also with other haptens having a different acid group in the meta position. If we make the difference greater by employing a hapten with the acid group in the para position, or use a hapten without any acid group, little if any cross-reaction is observed (Fig. 2-1). We conclude that antibodies are able to distinguish structural isomers (molecules containing the same groups but in different positions) but can also distinguish simple groups (acid groups in this instance) which occupy the same position.

It is a characteristic of antisera that the antibody molecules they contain are not all alike; they may differ in strength of reaction with a given hapten and may differ in their specificity. This is easily shown in the present case by allowing the anti-metanilic acid serum to react

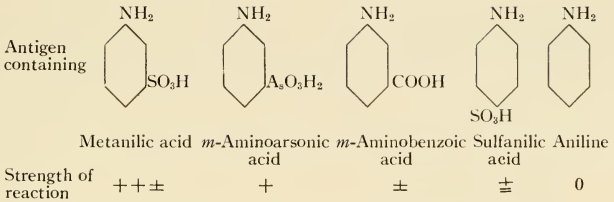


Fig. 2-1. Reaction of antiserum for metanilic acid (Landsteiner, 1945).

with a protein coupled with one of the cross-reacting haptens until no further reaction takes place, then testing the treated antiserum with the homologous and other related haptens. In order to avoid having soluble complexes of antibody and antigen left in the mixture, the anti-metanilic serum may be treated with haptens coupled to the insoluble structures (stromata) left after lysis of red blood cells and removal of the hemoglobin. A serum which has been thus treated to remove all the antibody which will react with a given antigen is said to have been absorbed with that antigen. The results of such an experiment (Landsteiner and van der Scheer, 1936) are shown in Fig. 2-2.

It can be seen that in each case absorption with heterologous hapten-protein compound, to the point of reducing the reaction with that hapten to zero, leaves considerable precipitating power for antigens containing the homologous hapten. Generally, it also leaves some reactivity for other heterologous haptens. Each hapten, evidently, combines with that fraction of the antibody molecules for which it has the highest affinity. The majority of the antibody molecules react best with the homologous hapten, most of which is left after heterologous absorption.

Similar results were obtained by Hooker and Boyd (1934) and Landsteiner and van der Scheer (1940) with egg albumins of various species, although here the exact nature of the chemical similarities which led to cross-reaction was not known.

From these and similar experiments (Landsteiner and van der Scheer (1936) drew the conclusion that "antibodies formed in response to one antigen, although adjusted to a certain structure, are not entirely uniform but vary in specificity to some degree." Boyd (1943)

Anti-metanilic acid(M) antiserum absorbed with azo-stromata made with:	Antigens made with chicken serum and: M	O	A	B
 (O)	++±	0	±	+
 (A)	+++	+±	0	+
 (B)	+++	+±	±	0
(Unabsorbed)	+++±	++	+	+±

Fig. 2-2. Precipitin reactions of absorbed anti-metanilic acid antiserum with various conjugated antigens.

suggested that we should think of the antibody molecules of an immune serum as "a large family, with varying degrees of deviation from a mean." Pauling, Pressman, and Grossberg (1944) made a similar and more precise suggestion. In their opinion, the free binding energies of the different antibody molecules (for the determinant that induced their formation) are distributed according to the Gauss error function.*

In the description of the reactions of antibodies with simple substances (haptens) it was stated that to detect anti-metanilic acid (anti-M) antibodies, for example, we make use of a protein, different from the one used as the carrier of the hapten during immunization, coupled with diazotized metanilic acid. It may have occurred to the reader to ask what would happen if we mixed the anti-M serum directly with metanilic acid?

* This is the well-known "normal distribution" formula of statistics,

$$f(x) = [1/\sigma\sqrt{(2\pi)}] \exp(-x^2/2\sigma^2)$$

where σ, called the standard deviation, is a measure of the "dispersion" or degree of heterogeneity of the population whose composition is summarized by the curve. Two graphic examples of this distribution are shown in Fig. 2-3.

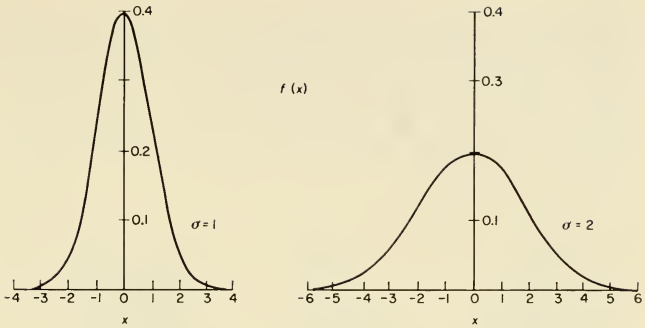


Fig. 2-3. Probability ("error") distribution when standard deviation equals 1 and 2.

The answer is that with simple haptens such as metanilic acid no visible reaction occurs as a rule. Originally, it was thought that the simple hapten was too small to take part in a precipitin reaction, but we are now inclined to believe that, although size may have something to do with it, the main deficiency of metanilic acid and other simple haptens in this respect is that they have only one point of attachment (combining group) for the antibody. Haptens containing two or more combining groups sometimes precipitate with the anti-hapten antibody.

Nevertheless, the anti-metanilic acid antibody has a strong affinity for metanilic acid, for it combines with this hapten when it is part of a metanilic acid-protein compound and forms a specific precipitate in the usual way though it does not precipitate metanilic acid from simple solution. The anti-M antibody may even combine with metanilic acid itself without producing a precipitate.

Inhibition Reactions

Now it was known that an excess of antigen usually prevents the production of a specific precipitate or greatly diminishes the amount. Clearly it must do this by combining with the antibody, for addition of an unrelated antigen has no such inhibitory effect. Therefore, Landsteiner reasoned that an excess merely of the hapten, which

logically must combine with specific antibody, ought in a similar way to prevent or diminish precipitation of the anti-hapten antibody with a hapten-protein compound. Experiments showed that this does in fact happen. Thus Landsteiner invented the inhibition reaction, which has been of enormous value in the study of immunochemical specificity. How specific inhibition works is shown schematically in Fig. 2-4.

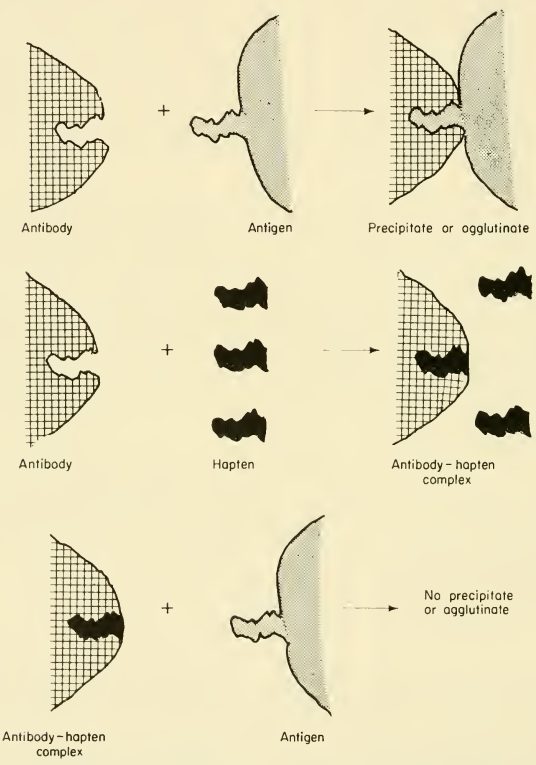


Fig. 2-4. Principle of inhibition by a hapten of serological reactions.

To inhibit completely the reaction of an anti-hapten antibody with the conjugated hapten-antigen generally requires a good deal more of the inhibiting hapten, in dissolved form, than that contained in the conjugated antigen. From this we might deduce that the binding force of the hapten alone with the antibody is less than the force which unites the antibody and the conjugated antigen. The hapten does combine with the antibody, however, for unrelated haptens have no inhibitory effect, and a given hapten does not inhibit unrelated antibody-antigen reactions. In other words, inhibition is specific. In Fig. 2-5 the inhibition is completely specific, that is, each hapten prevents precipitation only of the homologous antibody and antigen. That haptens combine with their specific antibody can be demonstrated by the power of antibody to prevent a diffusible hapten from dialyzing through a membrane otherwise permeable to it.

If closely related haptens are tested against the same antibody-

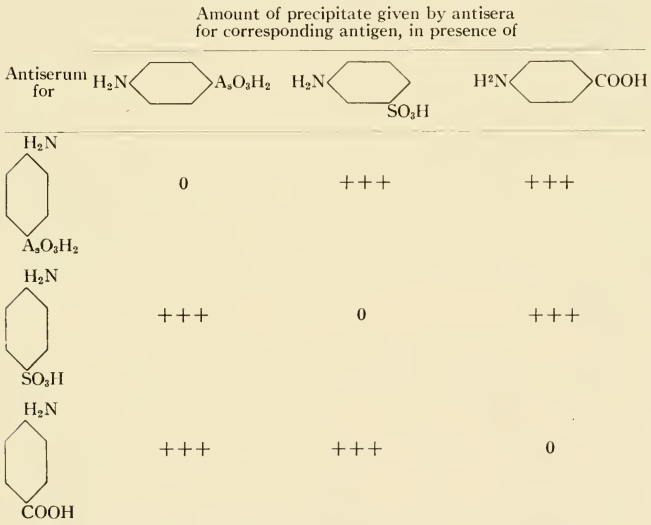


Fig. 2-5. Inhibition of precipitin reaction with homologous and heterologous haptens.

antigen system, it is usually found that the homologous hapten inhibits best and that other haptens inhibit more or less well, depending on the degree of their resemblance to the hapten contained in the immunizing antigen. Thus, in Table 2-1 we see that mononitrostrychnine inhibits the reaction of an anti-strychnine antiserum with a conjugated strychnine-antigen as well as does strychnine itself, whereas the related alkaloid brucine, which differs from strychnine

TABLE 2-1

Inhibition of Anti-Strychnine Sera by Various Haptens[a]
Hooker and Boyd, 1940

	Micromoles of test substance[b]								
Test substance	1.00	0.67	0.44	0.30	0.20	0.13	0.09	0.06	0.04
Strychnine			0	0	0	0	0	0	t
Mononitrostrychnine			0	0	0	0	0	0	t
Dinitrostrychnine								+	+
Monoaminostrychnine	0	0	0	0	0	0	t	t	+
Diaminostrychnine	0	0	0	0	0	0	0	t	t
Brucine			0	0	0	0	t	±	+
Morphine	+	+	+	+	+	+	+	+	+

[a] Hooker and Boyd, 1940.

[b] The symbols indicate the degree of precipitation obtained when the hapten-serum mixture was overlayered with a suitable concentration of the strychnine-protein antigen. The symbol + indicates a positive precipitation reaction, ± a weak reaction, "t" a faint trace, and 0 no precipitation. Absence of a symbol means that the test was not done or could not be read because of nonspecific precipitation.

only in possessing two methoxy groups, does not inhibit as well; that is, a larger amount of it is required to prevent the antibody-antigen reaction. The unrelated alkaloid morphine does not inhibit at all. In this case the effectiveness of different haptens was compared by testing decreasing amounts (increasing dilutions) of the haptens against a constant amount of antiserum, to which was later added a suitable amount of antigen.

In general, we may expect the results obtained with haptens H, H′, H″, and G, where H′ is closely related chemically to H, H″ less

closely related, and G unrelated, to give results similar to those in Table 2-1. This is shown schematically in Table 2-2.

TABLE 2-2

Precipitation Reaction of Anti-H Antibody and H Antigen, in Presence of Hapten*

	Dilution of hapten					
Hapten	1:2	1:4	1:8	1:16	1:32	1:64
H	0	0	0	0	+	+±
H′	0	0	±	+	++	++
H″	0	±	+	++	++	++
G	++	++	++	++	++	++

* ++ = Strong reaction. Other symbols as in Table 2-1.

The relative effectiveness of different haptens as inhibitors can also be shown by plotting concentrations, or maximum *dilutions* which inhibit (Fig. 2-6).

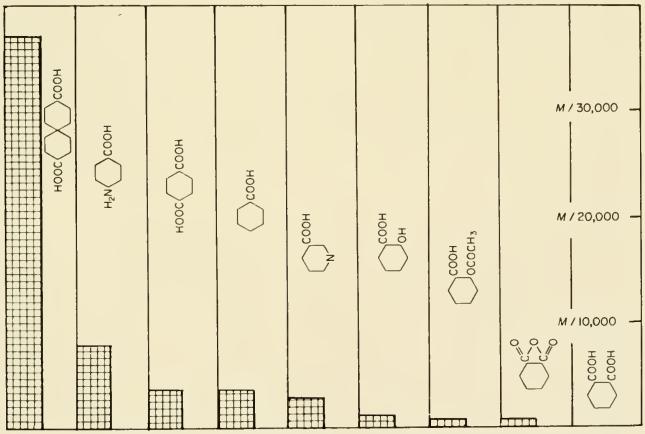


Fig. 2-6. Inhibition of precipitin reaction of anti-benzoic acid antibody by various haptens.

Another way of comparing the inhibiting powers of different haptens is to use constant concentrations (preferably expressed as molarities) of haptens against successive dilutions of the antiserum. In this case, again using the hypothetical haptens H, H′, H″, and G, we might obtain the sort of result shown in Table 2-3.

TABLE 2-3

Precipitation Reaction of Anti-H Antibody and H Antigen, in Presence of Hapten*

	Dilution of antiserum					
Hapten	1:2	1:4	1:8	1:16	1:32	1:64
H	+	0	0	0	0	0
H′	++	+	±	0	0	0
H″	++	++	+	+	±	0
G	++	++	++	++	+	±

* Symbols as in Table 2-2.

Instead of trying to find the antibody concentration which is completely inhibited by a given concentration of hapten, or the hapten concentration which will completely inhibit a given concentration of antibody, it is more accurate to measure the amount of precipitate produced under the various conditions, and estimate the amount of hapten which gives just 50 per cent inhibition.

Statistical Methods

If several such series of quantitative measurements are carried out, it is possible to obtain a mean (average) estimate of the 50 per cent inhibiting dose and, from the standard deviation of this mean, an estimate of its reliability. When such standard errors are calculated they tend to be rather large, for the quantitative precipitin technique is not as reproducible as the measurements of inorganic quantitative analysis or physical chemistry are. For this and a variety of other reasons, standard errors are not usually calculated for such estimates: (a) The necessary determinations would require too great an outlay of the experimenter's time and of an

antibody that may be in short supply. (b) The goal of such experiments is not usually an estimate of the actual inhibiting dose of any one particular hapten, but an estimate of the relative inhibiting power of two different haptens; in other words, a ratio. It is quickly found that attempts to calculate the standard error of a ratio from the standard errors of the two numbers involved leads one into Higher Statistics.

It might seem to the non-serologist that in the simple type of inhibition study shown schematically in Tables 2-2 and 2-3 statistical methods could be applied and would be helpful, but this is not generally the case either. To begin with, inhibition experiments are ordinarily interpreted as if inhibition were an all-or-none phenomenon. Thus from the first line of Table 2-2 we conclude that for complete inhibition (tube 4, counting from the left) of the amount of serum used in the experiment 1/16 of the amount of hapten H contained in a unit volume of stock solution is sufficient. But for all we know the amount of hapten in tube 4 may be anywhere from 1.02 to 1.98 times the minimal inhibiting dose (MID) of H. If tube 4 contains 1.02 MID, then tube 5 in turn contains only 0.51 MID, and the unavoidable accidental variations in experimental conditions are not likely to cause tube 5 to give a negative reading, though they well might make tube 4 positive. But if tube 4 contains 1.98 MID, which is equally possible, then tube 5 would contain 0.99 MID, and a slight variation in the conditions of the experiment might mean that tube 5 would read negative instead of positive. Thus in different experiments our estimate of the smallest amount of hapten that will completely inhibit a given amount of antiserum might vary from 1.0 to 2.0 to 0.5 mM. An experimenter is likely to feel that he is wasting his time in averaging numbers like 1.0, 2.0, and 0.5, not to speak of trying to estimate a standard deviation and a standard error of the resulting mean.

Also, it must be realized that, just as the results with hapten H might vary from 1.0 to 2.0 to 0.5 mM, so the results with hapten H′ might vary from 0.25 to 0.5 to 0.125 mM. It is expected that the variations in estimated MID's of the two haptens will generally go in the same direction; indeed, this is one of the reasons for running all the tests simultaneously, but it is apparent that the ratio of the apparent MID's *might* vary from 16 to 1.

In the second place, the all-or-none interpretation of inhibition experiments is an oversimplification. The experiment summarized in the first line of Table 2-2 actually yields more information than is contained in the mere statement that tubes 1 to 4 are negative and tubes 5, 6, etc., are positive. The strength of the reaction in the first tube to the right of the last negative tube also contributes information, for the reaction can vary from weak to strong. Taking the simple point of view, for example, we should estimate from Table 2-2 that hapten H′ is only one-fourth as effective an inhibitor as hapten H, for it takes four times as much to produce complete inhibition. But if we take account of the fact that the next tube after complete inhibition gives a reaction of + in the case of hapten H and only ± in the case of H′, it is clear that H′ is actually somewhat more than one-fourth as effective as H′. But how much more? It is hard to put such things into numerical terms. It is possible to invent codes for the translation of such readings into quantitative terms, or appropriate numerical scores may be found by statistical methods (see, for example, Fisher, 1950, pp. 289–295). In general, however, such treatments of the results of inhibition tests have not been found to extract enough extra information from the results to justify the calculations involved.

Recognizing, therefore, that the results of inhibition experiments are only semiquantitative at best, serologists who are attempting to compare the inhibitory power of two different haptens do not generally attempt to make quantitative estimates, but are content to say merely that hapten H is more effective, for this particular serum, than H′ is. Some tend to rely on the old rule of thumb, which is pretty well borne out in practice, that a difference in the results obtained with two haptens is significant if the difference in their inhibiting capacity differs rather consistently, from one experiment to another, by two tubes (ordinarily meaning a four-fold difference in effective concentrations). If the results do not differ by this much, one may suspect a difference in the effectiveness of the two haptens, without venturing a confident opinion. Even such a difference, however, arbitrarily judged to be "non-significant," may be of value as a guide to further experiments.

The value of statistical methods in general is of course not in doubt. For a long time certain biologists, and immunologists in

particular, often failed to avail themselves as fully as they might have of statistical methods (Batson, 1951). The situation has pretty well been corrected in recent years, however. A summary of some of the current applications of statistics to immunology will be found in my *Fundamentals of Immunology* (Boyd, 1956). Indeed, it is to be feared that today there are a few biologists who feel, as many physical anthropologists did 30 years ago, that statistics will cure all ills. Actually, of course, the results of statistical analysis can never be better than the data themselves. A false sense of security stemming from a blind application of statistical methods to situations where they are not appropriate can be as bad as the former tendency to avoid their use.

Stereoisomerism

Having shown that antibodies can distinguish structural isomers, Landsteiner naturally asked if they could also distinguish stereoisomers and therefore next turned his attention to this problem. In organic chemistry it is established that, whenever a carbon atom has four different groups attached to it, there are two possible arrangements of these groups which are essentially different from each other, somewhat as the right and left hands differ from each other (Fig. 2-7).

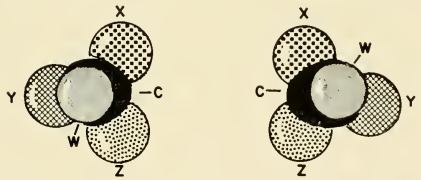
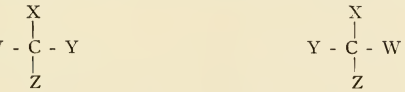


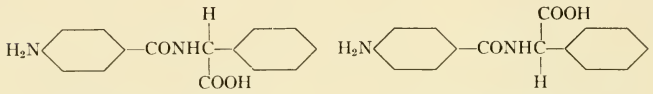
Fig. 2-7. Models of right- and left-handed molecules.

The essential difference between the two possible isomers in such a case is correctly shown only in three dimensions. Since it is not generally convenient to have three-dimensional models in front of us when discussing isomerism, it is customary to represent such compounds by a projection on two dimensions, as shown below.



As long as we remember that such projections must not be taken out of the plane of the paper, no incorrect conclusions will be drawn from their use.

Organic compounds differing in the spatial arrangement of the four different groups attached to a carbon atom have the same chemical and physical properties but differ in their effect on polarized light. Therefore, they are said to be optical isomers. An example of such an optically isomeric compound is aminobenzoylphenylaminoacetic acid, which exists in two forms. One, the *d* form, rotates the plane of polarized light to the right. The other, the *l* form, rotates it to the left (Fig. 2-8).



d-*p*-aminobenzoylphenylamino-acetic acid

l-*p*-aminobenzoylphenylamino-acetic acid

Fig. 2-8.

Containing an aromatic amino group, these compounds can be diazotized, coupled to proteins, and made to function as haptens. Since optical isomerism plays a very important role in biochemistry, we would expect that antibodies would be able to distinguish these two isomeric haptens. In fact, Landsteiner and van der Scheer (1928) found that, although the undiluted antigens gave some cross-reaction, they reacted quite specificially when diluted one to one hundred (1:100).

In later work Landsteiner and van der Scheer (1929) showed that D- and L-tartaric acid (Table 2-4), where two asymmetric carbon atoms are involved, could be differentiated by the appropriate antibodies and that both were distinguishable from the "internally compensated" mesotartaric acid.

Next to proteins, polysaccharides are the most important natural antigens. It was therefore logical to ask if isomers of sugars which differ in the configuration of one or more carbon atoms and are not necessarily optical antipodes could be distinguished by antibodies. Goebel and Avery (1929) showed that the monosascharides D-glucose and D-galactose, which differ only in the configuration of the fourth

TABLE 2-4

Serological Specificity of Stereoisomers of Tartaric Acid

	Antigen from[a]		
	l-Tartaric acid	*d*-Tartaric acid	Mesotartaric acid
	COOH | HOCH | HCOH | COOH	COOH | HCOH | HOCH | COOH	COOH | HCOH | HCOH | COOH
Immune serum for:			
l-Tartaric acid	+++	±	+
d-Tartaric acid	0	+++	+
Mesotartaric acid	±	0	+++

[a] Symbols indicate degree of precipitation when antisera for conjugated proteins containing isomers on left were mixed with proteins containing isomers shown on right. The symbol +++ indicates strong positive reaction; 0, negative reaction.

carbon atom (Fig. 2-9), could be distinguished serologically. Later work (Avery, Goebel, and Babers, 1932) even showed that the alpha and beta anomers of glucose, when converted to the *p*-aminophenyl-

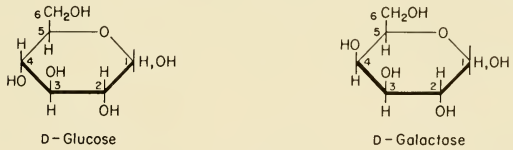


Fig. 2-9.

glucosides, diazotized, and coupled to a protein, gave rise to different antibodies (Fig. 2-10). The distinction was not as sharp as between

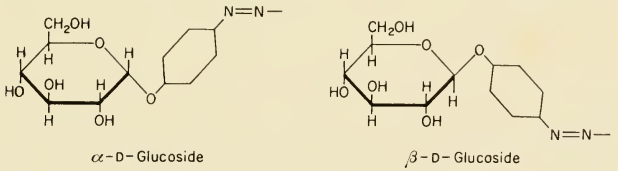


Fig. 2-10. α- and β-D-glucoside haptens.

glucose and galactose, which, in view of the fact that α- and β-glucose are spontaneously interconvertible in solution, seems reasonable.

Limitations of Specificity

It is apparent from this and similar work, that though the power of antibodies to distinguish small chemical differences in antigens is very considerable, this discrimination has certain limits, limits which, in the case of the alpha and beta anomers of glucose, we have almost reached. The number of different antibodies is certainly large, but we are moved to ask: Is there perhaps a limit to the number of substances which can be distinguished serologically?

We have by no means tested all possibilities, but I believe that the answer to the above question is that there probably is a limit. In the first place, cross-reactions are regularly found with closely related antigenic determinants (haptens), as we have just seen. In the second place, antibodies to natural antigens are not directed toward the molecules as a whole but toward relatively restricted portions of the molecule (Chapter 3). These restricted portions of the molecule consist of amino acid residues and combinations of amino acid residues in the case of proteins and, in the case of carbohydrates, of monosaccharide residues and combinations of monosaccharide residues. The number of amino acid residues occurring naturally is only somewhat greater than twenty (Yčas, 1958). The number of monosaccharide residues occurring in any considerable amount in nature is probably not much greater. The number of possible antigenic specificities is therefore not infinite. It is accordingly not surprising that, as more and more cross-reactions between antigens of unrelated or remotely related origins are tried, more and more cross-reactions are found to take place.

Thus, cross-reaction occurs between human blood group A substance and pneumococcus type 14 capsular polysaccharide (Finland and Curnen, 1940). Anti-pneumococcus type 14 sera strongly cross-react with a galactan isolated from cow lung (Heidelberger and Wolfram, 1954). Pneumococcus type 2 capsular polysaccharide and the polysaccharide from encapsulated type B Friedländer bacillus cross-react (Avery, Heidelberger, and Goebel, 1925). Highly active substances with specificity similar to that of the human blood group

substances are found in certain plants (Springer, 1958). One may predict that the number of such serological similarities will grow as the number of individual antigens tested for cross-reactivity increases.

Combining Groups of Antibody

The fact that no striking chemical differences between antibodies or between antibody and normal globulin have yet been found suggests that the portion of the antibody molecule responsible for its specific combining properties cannot be very large. This idea is supported by the evidence, to be discussed in the next chapter, that the portion of the antigen with which an antibody combines is relatively small, at least compared with the size of a protein molecule. From experiments of Landsteiner and van der Scheer (1938), Campbell and Bulman (1952) computed that the specific combining site of an antibody is not larger than 700 square angstrom units (700 A^2).

It is believed that van der Waals forces are among the most important in the union between antibody and antigen. Since these are very short range forces, being inversely proportional to the seventh power of the distance, the combining groups of antibody and antigen probably come into intimate contact to produce union as firm as that actually observed. (The free energy change $-\Delta F$ is of the order of

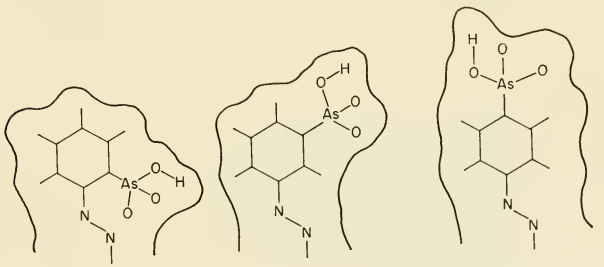


Fig. 2-11. Van der Waals outlines of *o*-, *m*-, and *p*-azobenzenearsonates. (From L. Pauling and H. A. Itano (eds.), 1957, *Molecular Structure and Biological Specificity*, American Institute of Biological Sciences, Washington, by permission of the editors and publishers).

5 to 9 kcal. per mole. See Chapters 9 and 10.) Hooker and Boyd (1941) suggested that the combining group of the antigen might fit into a cavity in the antibody. Pauling made a similar suggestion. Figure 2-11 shows Pauling's conception of antibody cavities corresponding to *o-*, *m-*, and *p*-aminoarsonic acid.

To what extent such a cavity in the antibody is merely schematic and to what extent it is real is not yet decided. The concept has certainly proved useful in thinking about antibody-antigen reactions.

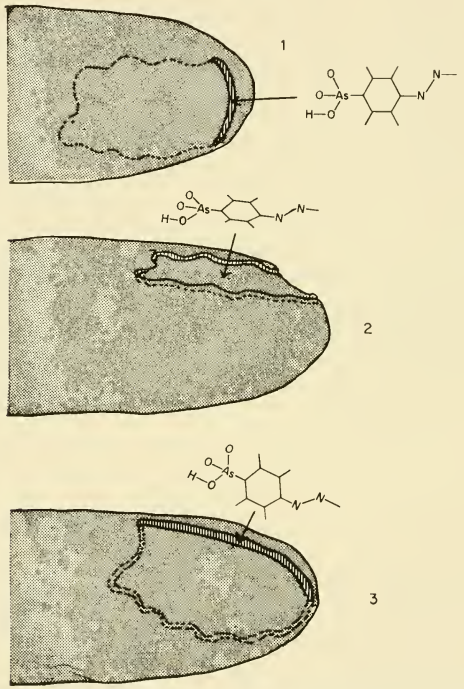


Fig. 2-12. Schematic drawings of three possible types of cavities (determinants) in antibody molecule: 1, invagination; 2, shallow trough; 3, slit trench.

In any case, the cavity is not necessarily the deep invagination suggested by Fig. 2-11 and example 1 in Fig. 2-12. The antigenic determinant might alternatively be accomodated lying on its side in a shallow trough (example 2 in Fig. 2-12), or sidewise in a sort of slit trench (example 3 in Fig. 2-12). According to Pressman (1957), there is evidence that all three types of antibody cavity exist.

If the combining group of an antibody molecule is relatively small (one such group, according to the above estimate, would amount to about 2 per cent of the surface of an antibody molecule), we naturally ask how many such groups an antibody has. It seems conceivable that one group would be enough to account for the reactions of antibody. For some time Dr. S. B. Hooker and I and some other workers in this field maintained that on the basis of economy of hypotheses (Occam's razor) it should be assumed that antibody was univalent. Others assumed that antibody was multivalent. There is now considerable experimental evidence indicating that neither party to this controversy was wholly right, for the valence of antibody seems to be two. There are certain antibodies, especially in connection with the Rh blood groups, behaving in peculiar ways which have led to their being described as "incomplete" or "univalent." The presently available evidence, however, indicates that the peculiarity of their behavior is not due to their having less than the usual number of combining groups but to other features of the molecule.

Formation of Antibody

We must now ask ourselves: How does the body manage to produce relatively large amounts of globulin molecules, so precisely adapted to combining with definite chemical groupings?

It is not easy to answer this question. A number of hypothetical mechanisms of antibody formation have been proposed, of which we may mention (a) the cast-off receptor theory of Ehrlich, (b) the template theory of Haurowitz, (c) the template theory of Pauling, (d) the "trained enzyme" theory of Burnet, and (e) the "natural selection" theory of Jerne, which is supported by Talmage and Lederberg.

(a) According to the theory of Ehrlich, antibodies are simply

natural preformed receptors of the body cell for various chemical groupings. When the number of such chemical groupings coming in contact with the cell is increased (antigenic stimulus), an excess of such receptors is formed. Some are cast off into the circulation and constitute circulating antibody. This theory was given up when it was found that antibodies could be formed against artificial groupings with which the organism had never come in contact in the course of its evolution and for which it could hardly be expected to possess preformed receptors.

(b) According to Haurowitz (1953), a template, which (as the result of the presence of a molecule of antigen) reflects in reverse the significant portions of the structure of the antigen held in the expanded configuration by polar forces of a molecule of nucleic acid, attracts to itself molecules of amino acids from which a duplicate of itself is built up and cast off into the circulation. This theory seems to require the persistence of small amounts of antigen throughout antibody formation, although this might not strictly be a necessary part of the theory.

(c) Pauling's theory (1940) is a modification of that of Haurowitz and differs mainly in Pauling's supposition that preformed normal globulin, becoming unfolded ("denatured") at the ends of the polypeptide chain (he assumes that they have accessible to them a number of about equally stable folded configurations), fold up (are "renatured") on contact with a molecule of antigen and thus become specific antibody. This theory definitely presupposes the persistence of antigen.

(d) Burnet and Fenner (1949) suggested that enzymes involved in the destruction of normal body constituents become adapted to acting on similar molecules of foreign substances, are self-reproducing, and continue to multiply after the elimination of the antigen. Antibodies are supposed to be enzymatically inactive partial replicas of these adapted enzymes. Burnet has apparently more recently changed his views (Burnet, 1957, 1959).

(e) Jerne (1955) suggested that globulin molecules of a very wide variety of configurations and therefore of specific reactivities are continually being produced by the body. Some of these molecules happen to have configurations complementary to surface groups of some antigens; these are the "natural antibodies." When an antigen

enters the circulation, it combines with those molecules which happen to have the corresponding specificity. These combinations are phagocyted and transported to the antibody-forming cells. There the antigen is dissociated and probably discarded, and the cell—for reasons not specified—proceeds to make more globulin molecules like those just introduced. The casting off into the circulation of these new specific globulins constitutes the phenomenon of antibody rise.

Jerne's theory, in spite of having been proposed only recently, has found considerable favor. Talmage (1957, 1959) considers it essentially similar to the theory of Ehrlich but suggests that the replicating elements are cells rather than extracellular protein. Burnet (1957) and Lederberg (1959) also support the theory. According to Burnet, antigen combines with specific receptors on the surface of lymphocytes and thereby stimulates these particular cells to settle down and multiply in an appropriate tissue. The result of this replication of selected cells is the production of more of the type of globulin molecule with which the antigen combined in the first place.

Burnet and Lederberg both assume that the antibody-forming cells are "hypermutable," i.e., that normally there are frequent changes in the types of globulin molecules a cell is genetically capable of producing. Thus, all possible types of gamma globulin molecules would generally be represented in the circulation *with the exception of* those produced by those cells that happened to combine with antigen while they were still immature; this is supposed to result in the elimination of such cells. This additional assumption is made to account for the nonproduction of antibodies to antigens of the body itself and for "acquired immunological tolerance."*

Any attempt to revive the Ehrlich theory must take account of the objection that antibodies can be formed to antigens for which the body can hardly be expected to have preformed natural receptors. Talmage (1959) tries to do this by supposing that sharp specificity, when observed, results from a mixture of globulin molecules, not all alike, each with some degree of specificity for the antigen or hapten. With the help of a diagram (Fig. 2-13) and by thermodynamic calculations he tries to show how the "information" and net specificity of

* When animals are injected with an antigen during fetal life, or in some cases shortly after birth, they may be incapable of responding immunologically to this antigen as adults (see Chapter 3).

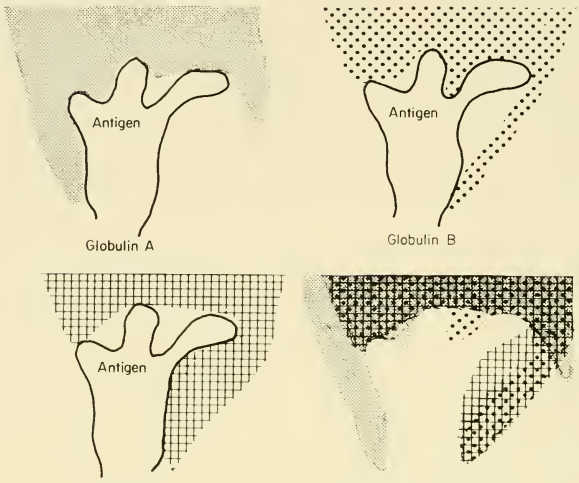


Fig. 2-13. Two-dimensional diagrams illustrating the concept that the information and net specificity of a combination of three different globulin molecules may be greater than that of one globulin alone. (Redrawn from Talmage 1959.)

a combination of different globulin molecules could be greater than those of any one type of globulin alone. Talmage suggests that the average "monospecific" serum contains ten to 100 different kinds of globulin molecules and points out that on such a basis the assumption of about 5000 different possible natural globulins could account for approximately 3×10^{120} different specificities. Since this number is larger than the number of electrons the universe is supposed to contain, Talmage believes it is satisfactorily large. In fact, Haurowitz (1956) estimated that not more than 50,000 different antibodies exist.

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CHAPTER 3

Antigens

Definition

We use the term antigen in at least two senses. Primarily, an antigen is a substance which, when introduced into an animal, usually not by way of the digestive tract, causes the production of specific antibodies. Immunologists commonly use the term antigen also for preparations which merely *react* with antibodies *in vitro*; for instance, the mixture of normal tissue lipids used in the Wassermann test for syphilis is referred to as the Wassermann antigen, although injection of it into an animal would probably not cause the production of syphilitic antibodies.

Immunologists are also imprecise in another way in their usage of the term antigen. The word is applied to both a purified, supposedly molecularly homogeneous preparation, as for example crystalline bovine serum albumin, which, when injected, will cause the production of antibodies to this substance, and also, following tradition, to preparations which, chemically speaking, are complex mixtures, such as the suspensions of killed organisms which are used in the practical production of certain types of immunity.

Antigenicity

In spite of many studies on the subject we are not yet in a position to state positively what physical and chemical characteristics make a substance antigenic. We can only offer certain rules of thumb: (a) antigens are substances the molecules of which are larger than mini-

mum in size, and (b) they must be foreign to the circulation of the animal in which they stimulate antibody production.

It is not possible to give a definite figure for the minimal molecular weight which a substance must possess to be an antigen, but good antigens generally have a molecular weight of not less than 10,000. It is also found that by adsorbing small molecules particulate material may become antigenic, just as conjugated antigens may be produced by coupling simpler compounds (haptens) to proteins. Some simple substances not conjugated to a protein cause the production of antibodies, but it is believed that they act by first combining with some of the proteins of the body.

Size alone does not seem to be enough. In addition to being large, a molecule, to be antigenic, must possess other characteristics. It has been suggested that a certain degree of internal complexity may be required, and it has been found that sulfonated polystyrene (Fig. 3-1), which is a large molecule polymer made up of a single

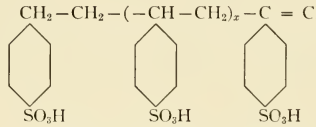


Fig. 3-1. Sulfonated polystyrene.

repeated unit, is not antigenic (Boyd, 1952). Haurowitz (1952) suggested that the necessary feature is a rigid structure of the determinant groups of the antigen. In support of this idea, it has found that gelatin, a non-rigid molecule, which is ordinarily a very poor antigen, can be made into a relatively good antigen by being coupled with chemical groupings which would be expected to increase the rigidity of the molecule (Hooker and Boyd, 1932; Clutton, Harington, and Yuill, 1938; Sela and Arnon, 1960). Contrary to earlier opinion, the introduced groups do not have to be aromatic (Sela and Arnon, 1960). Haurowitz's suggestion is also supported by studies on the antigenicity of synthetic polypeptides. Polyglutamic acid was found to be non-antigenic (Maurer 1957), and most of the polymers studied by Stahmann and his colleagues (references in Sela, 1962) were either non-antigenic or poor antigens. On the

other hand, Gill and Doty (1960) found a synthetic linear polymer containing tyrosine, which would increase the rigidity of the molecule, to be antigenic, and Sela (1962) found that a multichain copolymer, in which the chains of polypeptides containing L-tyrosine and L-glutamic acid were built on a multichain poly-DL-alanine, is a powerful and sharply specific antigen.

It was once believed that only proteins could be antigenic, but we now know that some carbohydrates are also good antigens. The rigid sites in polysaccharide antigens may be the pyranose or furanose rings (Sela, 1962). Large-molecule carbohydrates vary in their antigenicity. Pneumococcus polysaccharides are antigenic in man and in the mouse but not in rabbits (Dubos, 1945). Dextrans, apparently not antigenic for rabbits, are antigenic in man (Kabat and Berg, 1952, 1953). Purified blood group substances A and B are fair antigens in man but not in rabbits (Morgan and van Heyningen, 1944; Kabat, Baer, Day, and Knaub, 1950).

We repeat that substances must be foreign to the circulation to be antigenic for an animal. The normal animal does not produce antibodies to the protein and carbohydrate constituents of its own blood or to the tissue components which ordinarily reach the blood. Normal animals, however, can be induced to form antibodies to constituents of their bodies which normally do not find their way into the circulation, such as lens protein of the eye and casein, even from an animal's own milk (Lewis, 1934).

At one time it was believed that only proteins could be antigenic. We now know that many carbohydrates are also good antigens. Other classes of antigens exist, but with some possible exceptions all of them contain some protein or carbohydrate, or both.

Immunological Tolerance

For a long time it was a complete mystery why an animal did not make antibodies for the proteins and other substances of his own circulation, many of which are good antigens for an individual of a different species. A clue has recently been found in the phenomenon of immunological tolerance. If embryos are injected *in utero* or early in postnatal life with an antigen, not only may they not produce any antibody to the antigen, but they may be rendered incapable of responding to this antigen for the rest of their life (Burnet, 1956)

although they will generally respond perfectly normally to other antigens. This refractory state is called immunological tolerance.

To explain it and related phenomena, modern theories of antibody formation postulate, as was pointed out in Chapter 1, that the combination of antigen with immature antibody-forming cells results in the death of these cells or at least their elimination from the body. It seems likely that the mechanism that produces acquired immunological tolerance, whatever it is, accounts also for the failure of the body to produce antibodies to its own circulating antigens.

It is known that the rejection of tissue transplants from one individual to another, in contrast to the acceptance of transplants from another part of the patient's body or from an identical twin, is an immunological phenomenon. Acquired immunological tolerance has been strikingly demonstrated in animals by injecting adult tissue cells into embryos. Such injected embryos, when they are born and grow up, may accept skin grafts, for example, from a donor of the stock which provided the injected tissue, something they would not do if not previously injected during fetal life. Billingham, Brent, and Medawar (1953) believe that in such cases some of the injected cells have survived in the recipients, thus accounting for the continued receptive state for transplants from that stock.

Autoimmunization

Physiological mechanisms, like other machinery, can go wrong. It is therefore not too surprising that occasional individuals are suspected of producing antibodies to their own antigens. This process is called autoimmunization, and naturally it is not a good thing when it occurs. In fact, the process may be part of the etiology of a number of hitherto mysterious diseases, most of them fortunately rare, such as acquired hemolytic anemia, idiopathic thrombocytopenic purpura, chronic leukopenia, periarteritis nodosa, lupus erythematosis, and possibly other diseases.

The production of autoantibodies is made possible by a number of abnormal factors, which may include (a) modification of one of the patient's own antigens by combination with a drug, a bacterial toxin, or something of the sort, so as to make it at least partially "foreign" to his circulation, and (b) an unusual propensity of the patient to form antibodies in general.

Antigenic Determinants

Antibodies combine with the surface of antigenic cells or molecules. Even an antigen molecule of only moderate size can combine with several molecules of antibody. This shows that the portion of the antigenic surface toward which the antibody is directed (antigenic determinants) is only part of the whole molecule. In Chapter 1 we saw that antibodies can be produced which combine specifically with relatively small molecules such as arsanilic acid or glucose. The question arises: How much of the surface of a antigen molecule is actually involved in the combination with antibody?

Some information on this question has been obtained, mostly by use of Landsteiner's inhibition technique. Landsteiner (1942) found that hydrolysis products of silk, peptides with molecular weights about 600 to 1000, were capable of specifically inhibiting the reactions or precipitin sera for silk. This work has recently been confirmed by Cebra (1961), who found that tyrosine forms an important part of the antigenic determinant in silk fibroin, but that a considerable length of the glycyl-alanyl chain is also required for detectable specific combination. Dodecapeptides (MW ca. 900) were the most active of the peptides compared, giving up to 50 per cent inhibition. Of the octopeptides tested, Gly (Gly_3, Ala_3) Tyr (MW ca. 600) was the most effective inhibitor and probably represents a major part of the specific antigenic determinant.

Better evidence comes from reactions with conjugated antigens containing complex haptens. From quantitative studies of the inhibition of antibodies to simple haptens Hooker and Boyd (1933, 1941) concluded that the specificity of the antibody was influenced to some extent by the protein tyrosine or histidine residues with which the diazotized amines combine. This suggested that the antigenic determinant in conjugated antigens is not quite as simple a structure as the hapten alone (Fig. 3-2). Landsteiner (1945) studied the question by coupling to proteins haptens containing peptides made up of several amino acids. Goebel, Avery, and Babers (1934) and Kabat (1957) investigated antibodies directed toward determinants consisting of several sugar molecules linked together to form an oligosaccharide. Let us review some of these experiments briefly.

Peptide Determinants. Since some proteins are made up entirely of amino acids, and since there is no evidence that the specificity of

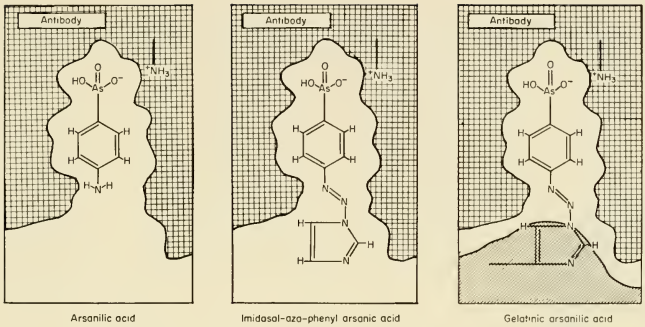
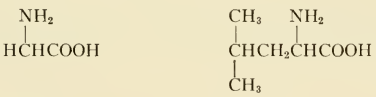


Fig. 3-2. Reactions of anti-gelatin-arsanilic acid antibody with various substances.

proteins containing a small percentage of carbohydrate is affected in any way by the presence of the carbohydrate, we are forced to conclude that the specific antigenic determinants of protein antigens consist of various combinations of amino acids. Anything we can find out about the specificity of peptides might therefore apply also to native proteins.

Landsteiner prepared *p*-aminophenyl compounds containing peptides made up of the amino acids glycine and leucine



Glycine Leucine

in various combinations (Fig. 3-3). These haptens were diazotized and coupled to protein in the usual way, using one protein for the immunizing antigens and another for the test antigens to avoid complicating cross-reactions due to antibody to the protein part of the antigens.

The strongest precipitation was obtained with the homologous antigen (Table 3-1), but cross-reactions were also obtained, generally strongest when the terminal amino acid of the peptide in the test antigen was the same as that in the immunizing antigen. Since these

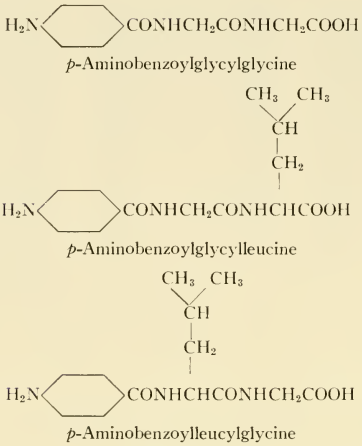


Fig. 3-3.

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haptens had an amino acid —COOH at the end, Landsteiner interpreted this as showing the predominant influence of the acid-carrying group on specificity. Today we are more inclined to attribute Landsteiner's cross-reactions to the fact that the terminal unit, acid or not, of a composite hapten has greater influence on the specificity than any other group does; for, as we shall see, a similar rule applies to haptens consisting of oligosaccharides where no acid group is present.

TABLE 3-1

Cross-Reactions of Glycine and Leucine Haptens[a]

	Antigen containing[b]									
Antibody for	G	L	GG	GL	LG	GGG	GGL	GGGG	GGGL	GGGGG
GGGGG	0	0	±	0	0	+±	0	+±	0	+±
GGGGL	0	+	0	+±	0	0	++	0	++	0

[a] Landsteiner, 1945.
[b] G = glycine, L = leucine.

When Landsteiner tested antibodies to larger peptides, he still found that cross-reactions occurred with peptides having the same terminal amino acids, but such cross-reactions did not always occur, and some cross-reactions were found to be due to common amino acids in other positions. The cross-reactions were definitely related to similarities of constitution (Table 3-1). For instance, an antibody for the pentapeptide GGGGG, where G stands for glycine, precipitated —GG but not —LG antigen, where L stands for leucine, and precipitated much less —LGG than —GGG. The amount of precipitate produced by an anti-GGGGL antiserum with various peptide-containing antigens increased in the order —L, —GL, —GGL, —GGGL, —GGGGL (Table 3-2).

TABLE 3-2

Increase in Strength of Cross-Reactions with Increase in Length of Hapten[a]

	Antigen containing[b]				
Antibody for	L	GL	GGL	GGGL	GGGGL
GGGGL	++	+++	+++	++++	++++

[a] Landsteiner, 1945.
[b] G = glycine, L = leucine.

The strongest reactions were not always obtained with haptens having the terminal portions identical with those of the immunizing hapten. For instance, when Landsteiner prepared antisera against polypeptides in which the terminal carboxyl group had been converted to the amide (Fig. 3-4) he found than an antiserum for

Fig. 3-4. Amide of *p*-aminobenzoylglycylglycine.

GGLGGAm reacted with —GGLAm and —GGGGLAm but not with —LGGAm (Table 3-3), in spite of the fact that the terminal three units of this last hapten are identical with the terminal three units of the immunizing hapten. Landsteiner attributed this to a

TABLE 3-3

Cross-Reactions of Glycine-Leucine-Amino Polypeptides[a]

	Antigen containing[b]			
Antibody for	GGLGGAm	GGLAm	GGGGLAm	LGGA
GGLGGAm	++±	+±	+±	0

[a] Landsteiner, 1945.
[b] G = glycine, L = leucine.

failure of the amide groups to have as strong an effect on serological specificity as the free carboxyl groups have.

Landsteiner obtained evidence that the antibodies to such complex peptide haptens were at least partly directed toward the whole peptide and not merely to the component amino acids. For one thing, varying the order of the amino acids in the peptide made a marked change, so that —GGL, —GLG, and —LGG were serologically different, as were —GGGGL, —GGGLGG, and —LGGGG.

Other evidence that antibody is directed toward the whole peptide was obtained by "absorbing" an antiserum, i.e., by reacting the antiserum with heterologous antigens until no further precipitate formed, and then reacting the absorbed antiserum with hapten. Suitable absorption of an antiserum for GGLGG left antibodies which reacted with the homologous hapten but not with related haptens, except for a slight reaction with —LGG. Tests made for comparison with diluted antiserum showed that this change in reactivity was not due merely to diminution in total antibody content.

A third line of evidence came from inhibition experiments. Landsteiner found that antibodies to a given peptide were generally better inhibited by homologous than by heterologous hapten, even when they reacted with a heterologous antigen (Table 3-4).

From the evidence that antibodies can be directed toward the whole of a peptide containing as many as five amino acids we may conclude that the antigenic determinants in natural proteins may be as large as this. Nevertheless, there seems to be a limit to the size of the antigenic determinant to which the combining group of a single antibody molecule can be directed, for Landsteiner and van der Scheer (1938) found that when they used symmetrical aminoiso-

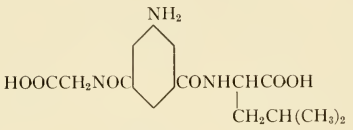
TABLE 3-4

Inhibition of Heterologous Reaction by Homologous Hapten[a]

Antibody for	Antigen containing	Reaction in presence of hapten[b]		
		GGG	GGGGG	GGLGG
GGG	GGG	±	±	+±
GGGGG	GGG	+	±	++
GGLGG	GGG	±	±	0

[a] Landsteiner, 1945.
[b] G = glycine, L = leucine.

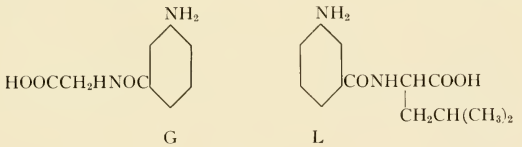
phthalyl glycine-leucine (Fig. 3-5), which they referred to as GIL, as hapten they obtained two distinct antibodies. One reacted with *m*-aminobenzoyl glycine (G) and the other with *m*-aminobenzoyl



GIL

Fig. 3-5.

leucine (L) (Fig. 3-6). The anti-G of such a serum was not removable with antigen containing only L, and the anti-L was not removable with antigen containing only G. Evidently the two amino



G L

Fig. 3-6.

acid residues in GIL were too far apart to be spanned simultaneously by a typical antibody determinant, although there was some evidence for the presence in the antiserum of a slight amount of a special

Carbohydrate in test antigens	Antibody to: α-glucoside antigen	β-glucoside antigen	β-galactoside antigen	β-cellobioside antigen
α-glucoside	+++	+		±
β-glucoside	+	+++	0	++
β-galactoside		0	++±	0
β-cellobioside	0	++	0	+++±
β-maltoside	++	0	0	±
β-gentiobioside	0	++	0	++
β-lactoside	0	0	++	±

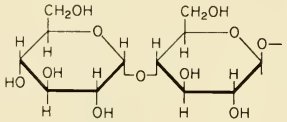
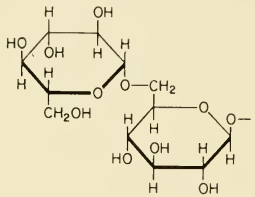
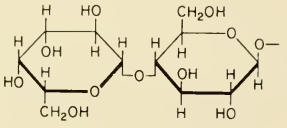
Carbohydrate in test antigens	Antibody to: β-maltoside antigen	β-gentiobioside antigen	β-lactoside antigen
			
α-glucoside	++	±	0
β-glucoside	++	++	0
β-galactoside	0	0	+
β-cellobioside	+±	++	++
β-maltoside	++++	+±+	±
β-gentiobioside	+	+++	±
β-lactoside	±	±	+++

Fig. 3-7. Reactions of antisera to synthetic antigens containing various mono- and disaccharides (Avery, Goebel, and Babers, 1932; Goebel, Avery, and Babers, 1934).

antibody which might have been directed toward the whole hapten GIL. At the same time, evidence was obtained that the anti-G and anti-L of the antiserum produced by injecting the GIL antigen were not quite identical with those produced by injecting G- and L-coupled antigens.

Carbohydrate Determinants. Experiments with carbohydrate haptens have given similar results. Goebel and co-workers (Avery, Goebel, and Babers, 1932; Goebel, Avery, and Babers, 1934) injected conjugated antigens containing monosaccharides and disaccharides as haptens (Fig. 3-7). Coupling was done by way of an aminophenyl group in each case. It will be seen that the cross-reactions of the antisera to the disaccharide haptens occurred mainly with the terminal sugars. Inhibition experiments showed that the *p*-aminophenyl glycosides of the terminal sugars (monosaccharides) were nearly as good inhibitors of the anti-disaccharide sera as the *p*-aminophenyl glycosides of the disaccharides themselves were. This again points to a predominant influence of the terminal group of a composite hapten on the antibody produced when an antigen containing it is injected. Nevertheless, the fact that the *p*-aminophenyl glycosides of the disaccharides were still somewhat better than the disaccharides alone as inhibitors of their corresponding antisera suggested that the anti-disaccharide antibodies were to some extent directed toward the whole hapten and that a carbohydrate hapten could be larger than a disaccharide.

Kabat (1957) was able to obtain further information on this point by studying the antibodies produced in human beings by injections of dextran, a large-molecule polysaccharide produced by certain bacteria. Dextran appears to be made up entirely of glucose, predominantly connected by 1-6 linkages (Fig. 3-8). With such a simple antigen the possible antigenic determinants are merely one or more glucose units. Finding out how big an antigenic carbohydrate determinant may have a specifically corresponding antibody determinant is simply a matter of finding out how many glucose units an oligosaccharide must contain to fill the combining site on the antibody. Kabat studied this question by measuring the relative inhibiting power of glucose, isomaltose (two glucose units), isomaltotriose (three glucose units), and larger polysaccharides for an anti-dextran serum acting on dextran. The results are shown in Fig. 3-9. It is

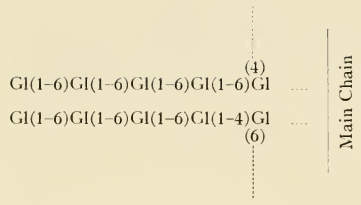


Fig. 3-8. Suggested structure of dextran.

apparent that isomaltose (two glucose units) is distinctly better than glucose as an inhibitor but that isomaltotriose is much better than either, suggesting that the antibody determinant corresponds to an antigenic determinant of at least three glucose units. Actually, the data suggest that the antibodies can distinguish even isomaltohexaose (six glucose units) from any smaller antigenic determinant, but the difference between the pentaose and the hexaose is not great. Kabat suggests that the hexaose is the largest group capable of entering the cavity in the anti-dextran antibody molecule.

If the hexasaccharide is accepted as the largest group which can totally combine with the combining site of the antibody, the contribution of each glucose residue, starting with the terminal unit, to the total free binding energy between antibody and antigen can be computed. Such calculations are shown in Table 3-5, taken from

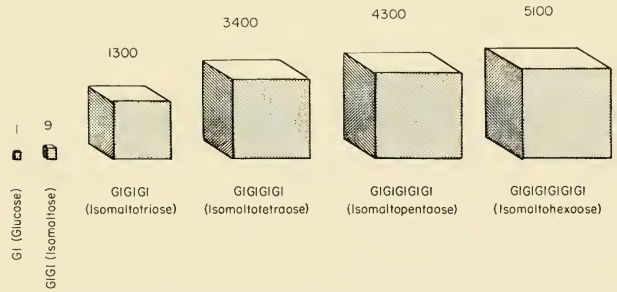


Fig. 3-9. Relative inhibitory power of oligosaccharides for anti-dextran serum acting on dextran. (Redrawn from data of Kabat, 1957.)

TABLE 3-5

Calculated Contribution to the Free Energy of Combination of Glucoses in Reactive Groups of Dextran[a]

Number of glucose units	Contribution to binding energy, %
5	98
4	95
3	90
2	60
1 (terminal unit)	39

[a] Kabat, 1957.

Kabat (1957). It will be seen that the terminal glucose contributes as much as 39 per cent of the total binding energy. The first five units together contribute 98 per cent of the binding energy, leaving only 2 per cent to be contributed by the sixth glucose residue.

These calculations give us a fairly good idea of the size of the antigenic determinant in a typical carbohydrate antigen. We shall see later how this information can be applied to practice.

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CHAPTER 4

Blood Groups

ABO Blood Groups

It does not take profound knowledge of science to realize that no two human beings, with the possible exception of identical twins, are exactly alike. There are sometimes strong resemblances in families, and sometimes even unrelated persons look enough alike to be mistaken one for the other by those who do not know them well, but close associates are very seldom deceived. Features, voice, movements, and modes of response nearly always distinguish each human being from all others in the world.

If we believe, and the belief hardly needs defending today, that structural and functional differences between individuals, aside from the effects of accidents resulting in scars or deformity and from learned behavioral patterns, are due to underlying biochemical differences, we should not be surprised to find that between different individuals of the same species biochemical differences can also be demonstrated. Surprisingly enough, this was done for the first time at the beginning of the present century. Karl Landsteiner, then working in Vienna, discovered that not all normal human blood is alike. Landsteiner and his pupils showed that human beings could be classified into four groups on the basis of the reactions of their blood with that of other normal individuals. This discovery made blood transfusion a safe and practical procedure for the first time and had great influence on the study of serological specificity.

Landsteiner's discovery consisted of the observation that, when the bloods of certain individuals were mixed, the red blood cor-

puscles adhered to each other and formed clumps that under the microscope looked something like bunches of grapes. (See Fig. 1-2, p. 3). In strong reactions all the red cells in the preparation stuck together, leaving a clear supernatant fluid. Landsteiner showed that this behavior could be explained by assuming that there may be two reactive substances in the erythrocytes and two corresponding anti-substances in the plasma which react with the erythrocyte substances. The substances in the erythrocytes have been shown to be antigens; they are also called isoagglutinogens. The substances in the plasma or serum which combine with them and thus cause agglutination have all the properties of agglutinating antibodies, and are called isoagglutinins.

It is obvious that the substance in the plasma which combines with the erythrocytes of another individual and causes them to agglutinate could hardly coexist in the same blood stream with the corresponding agglutinogen; for, if it did, an individual's plasma would agglutinate his own erythrocytes. The rule, first stated by Landsteiner and known by his name, is that those agglutinins will be present which can coexist with the agglutinogens present in the cells. The agglutinogens, called arbitrarily A and B, can be present in the cell singly or together, or can both be absent. This gives us the combinations of the four classical blood groups. (Table 4-1).

Landsteiner's discovery explained why transfusions of blood from one individual to another had previously only occasionally been successful. If a blood donor is selected at random, the chances of obtaining one whose blood group is compatible with that of the recipient are not good. It was only when pretransfusion blood grouping became a routine that blood transfusion became a safe and reliable procedure.

TABLE 4-1

The Landsteiner Blood Groups

Blood group	Antigens in cells	Agglutinins in plasma
O	—	Anti-A and Anti-B
A	A	Anti-B
B	B	Anti-A
AB	A and B	—

If a donor of the same blood group as the recipient is chosen, it is obvious that the chances of a successful transfusion are good, unless other blood factors yet to be discussed come into operation, or unless the technique is faulty. Transfusion consists in introducing a relatively small amount of the donor's blood, 500 ml. or less, into the circulation of the recipient. This means that the donor's cells are exposed to a large amount of whatever agglutinins the recipient possesses, in full concentration. If the donor's cells contain an agglutinogen capable of reacting with the recipient's agglutinin or agglutinins, the donor's cells may be agglutinated, and a serious or even fatal transfusion reaction may result.

It is not, however, always necessary to use a donor of exactly the same blood group as the recipient. Introduction of a donor's agglutinin which could react with the recipient's cells is often not serious, for the agglutinin gets diluted by the recipient's plasma and is also partly neutralized by soluble blood group substance in the recipient's plasma and in his tissues. This means that, in general, transfusions in the directions shown in Fig. 4-1 are possible, although it is always preferable to use a donor of the same group as the patient.

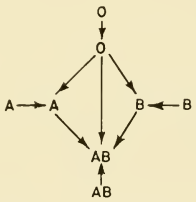

Fig. 4-1. Theoretical possibilities of transfusion, based on blood groups of donor and recipient.

Blood groups are inherited. Parents with any given combination of blood groups may produce children of certain blood groups but not of others, except that the mating of A $\times$ B may produce children of any of the four groups. Inheritance is based on three allelomorphic genes, A, B, and O, which can occur in any combination of two: OO, AA, AO, BB, BO, or AB. The blood groups of individuals of genotype AO are the same, so far as we can tell in the laboratory,

as those of individuals of genotype AA. The same holds for BB and BO. Consequently, we have to classify both AA and AO as group A, and BB and BO as group B, giving the four Landsteiner blood groups, as shown in Table 4-2.

TABLE 4-2

Genetically Determined Types (Genotypes) and Serologically Determined Types (Blood Groups)

Genotype	Blood group	Approximate percentage of U.S.A. population
OO	O	45
AA, AO	A	42
BB, BO	B	10
AB	AB	3

It was soon found that there were two kinds of A antigen. The more common one, and in Asian populations the only one present, reacts strongly with anti-A agglutinins and is designated as A_1. The other, confined to Europeans and Africans and their descendents in other parts of the world, often reacts weakly with anti-A and is called A_2. This distinction enables us to divide the population of Europe and Africa into six blood groups instead of four, as follows: O, A_1, A_2, B, A_1B, and A_2B. The difference seems to be of little importance for transfusion but is interesting to anthropologists and students of legal medicine.

Although in Table 4-1 group O erythrocytes are shown as having no antigen, this is not strictly true. They possess antigens connected with other blood group systems still to be discussed and also have an antigen connected with the ABO blood group system. Human plasma does not ordinarily contain an agglutinin for this antigen, but the plasma of individuals of the subgroup A_1B and the normal serum of certain animals may contain one. The agglutinin can be found in the serum of certain eels, apparently more regularly in the European than the American eel, and may be produced by immunizing a goat with *Shiga* bacilli. None of these sources is always available, nor is the agglutinin so obtained always strong and reliable. It

was therefore a considerable advance in blood grouping technique when it was discovered that saline extracts of the seeds of certain plants, such as *Ulex europeus,* which grows wild in Western and Southern Europe and in North Africa, contain an agglutinin specific for this antigen of the group O erythrocytes (Cazal and Lalaurie, 1952; Boyd and Shapleigh, 1954a). This plant agglutinin has apparently replaced all other reagents in this application.

At first it was believed that the agglutinogen detected by this agglutinin, whatever the source of the agglutinin, was an O antigen which had the same relation to the O gene as the B antigen has to the B gene. The agglutinin was therefore called anti-O. It was soon found, however, that erythrocytes of the subgroups A_2 and A_2B are also agglutinated by the agglutinin, A_2 cells being affected about as strongly as O cells. This is apparently true even when the genotype of the A_2 individual is A_2A_2, so that no O gene is present. It seemed improper to retain the name anti-O for a reagent that detects an antigen produced by both the O and A_2 genes. Following the practice of Morgan (Morgan and Watkins, 1948), the term anti-H is now generally used for the agglutinin and the term H for the antigen it detects.

Taking account of this and other discoveries about the blood groups, we may revise Table 4-1 (see Table 4-3).

TABLE 4-3

Subgroups of Landsteiner Blood Groups

Blood group	Subgroup	Antigens in cells	Agglutinins in plasma
O	O	H	Anti-A, anti-A_1, anti-B
A	A_1	A_1	Anti-B (sometimes anti-H)
	A_2	A_2 + H	Anti-B (sometimes anti-A_1)
B	B	B	Anti-A, and anti-A_1
AB	A_1B	A_1 + B	(Sometimes anti-H)
	A_2B	A_2 + B	(Sometimes anti-A_1)

Secretors and Nonsecretors

The antigens of the ABO blood group system are not confined to the erythrocytes. They may occur in practically all tissues and fluids of the body, with the probable exception of the central nervous sys-

tem. They may occur in two forms: water soluble and lipid soluble (i.e., soluble in lipid solvents such as alcohol-ether mixtures and chloroform). All individuals apparently have the lipid-soluble form in their tissues, in conformity with their blood group (Boyd and Boyd, 1937). The water-soluble form, however, is found in only about 85 per cent of European individuals. Such persons are called secretors, and those in whose tissues and body fluids water-soluble antigens corresponding to their blood group are not found are called nonsecretors (Schiff and Sasaki, 1932). The ability to secrete the A and B antigens in water-soluble form is inherited, being controlled by a pair of genes S and s.

The saliva of all secretors, no matter what their group, contains enough of the H antigen to make it possible to diagnose such individuals by the inhibition technique with an anti-H reagent such as Ulex extract (Boyd and Shapleigh, 1954b). Saliva of group O secretors is richest in H antigen, and, according to Race and Sanger (1958), some A_1B salivas may not contain enough H antigen to make the use of Ulex extracts reliable for the diagnosis of secretors in this subgroup.

The above sketch does not by any means give an adequate picture of the ABO blood group system, which is one of the most complicated known in man. A good discussion is given in the book by Race and Sanger (1948). We may simply mention that a number of other variants of the A antigen are known, all of them fortunately rare, and that genes exist, also rare, which can modify the expression of the ABO genes. The "Bombay" gene, x, when present in double dose, xx, prevents the development of antigens B and H; whether it also suppresses A is not yet known. There seems to be another gene, y, which when present in double dose, yy, modifies the development of the A antigen in the red cells and, to a much lesser extent, in the saliva. Variants of the B antigen have also been observed.

Blood Groups of the Ancient Dead

Antigens A and B are much more stable than most protein antigens. (The chemical nature of the A and B antigens will be discussed in Chapter 7.) It is comparatively easy to demonstrate A and B in dried tissue, boiled erythrocytes, or tissues which have been preserved in formaldehyde. These facts led Boyd and Boyd (1934,

1937) to attempt to demonstrate A and B in mummified human remains. The attempt seemed to be successful. These workers tested more than 300 specimens, mostly from Egypt in the Old World and from Mexico and Peru in the New World. The technique, though exacting and at times even exasperating, was simple in theory; pulverized dried tissue (usually muscle) was mixed with carefully titrated anti-A and anti-B agglutinins and the mixture tested after a suitable interval for evidence of removal of one or more of the agglutinins. Removal of anti-B was considered to indicate the presence in the tissue of the B antigen, removal of anti-A the presence of A. Removal of neither suggested either that the specimen came from an individual of group O or that any antigens originally present had deteriorated. Removal of both anti-A and anti-B suggested group AB or nonspecific destruction or removal of agglutinins or antibodies in general.

The results obtained were on the whole in line with the present distribution of the A and B antigens in human races, confirming the antiquity of the ABO blood group system. (Some authors had suggested, amazingly, that the A and B genes were of recent origin.) The B antigen was apparently found in pre-Columbian specimens from Mexico (Taylor and Boyd, 1943), a finding which, if ever confirmed, might support the suggestion, made on other grounds, that the B gene was eliminated in the aboriginal inhabitants of America by natural selection (Boyd, 1959). This subject has been reviewed by Smith (1960).

Origin of Isoagglutinins Anti-A and Anti-B

The presence of anti-A and anti-B in normal human plasma seems at first glance to be an exception to the rule that antibodies to blood group antigens do not usually appear without some history of unusual antigenic stimulus. The exception is a marked one, for the occurrence of these isoagglutinins is very regular. When anti-A or anti-B, if expected according to Landsteiner's rule, are absent, there is usually a special explanation, as Race and Sanger point out. It is natural to ask why these agglutinins appear with such regularity. There have been two main theories.

According to one theory, the isoagglutinins anti-A and anti-B are a result of the action of the ABO genes just as the ABH antigens are.

This theory has been supported by Furuhata (1927) and the Wurmsers (Filitti-Wurmser et al., 1954). Whether this theory seems plausible depends partly on which theory of antibody formation we happen to believe.

Another theory suggests that anti-A and anti-B are immune antibodies, as most other agglutinins are, being formed in response to antigens, in food, in bacteria, and in animal parasites, which are chemically similar to the A and B antigens of man. It is known that a number of such related antigens exist; in Chapter 2 the cross-reaction of blood group A antigen and type 14 pneumococcus was mentioned.

If the second theory of isoagglutinin formation is correct, one wonders why natural isoagglutinins for other human blood antigens, such as M, N, and Rh, are so seldom encountered. One possible reason is the lower antigenicity of these agglutinogens; some evidence for this exists. Another reason might be that the human agglutinogens M, N, and Rh are more unusual in their structure than A and B, so that closely related antigens in lower organisms and in food, serving as stimuli for the formation of anti-M, anti-N, and anti-Rh antibodies in man, are only rarely encountered. Later on I shall mention some recent evidence in support of this speculation.

MNS Blood Groups

Although unknown for so long, the existence of the ABO blood groups was relatively easy to demonstrate because of the normal presence of the isoagglutinins anti-A and anti-B. However, over a quarter of a century went by before any other blood group system was discovered in man. In 1927, Landsteiner and Levine demonstrated the existence of M and N antigens (also P) by the use of absorbed sera of rabbits injected with human erythrocytes. Because anti-M and anti-N isoagglutinins rarely occur and because patients are not readily immunized to these antigens, they have little importance for transfusion; their applications have been mainly to legal medicine and to anthropology.

A number of variants of the M and N antigens and, in addition, other antigens associated with the M and N factors in inheritance have been discovered. Two of these, S and s (to be distinguished

carefully from the Ss gene pair which controls the secreting phenomenon in the ABO system) are fairly common nearly everywhere and add greatly to the anthropological usefulness of the system. Two others, called Hunter and Henshaw after the donors in whom they were first found, are not too common in Africans and are virtually unknown in persons of European descent.

Distribution of M and N in the Body

Boyd and Boyd (1934) were not able to demonstrate M and N in human tissues with the technique which they had devised for A and B. Kosyakov and Tribulev (1939, see also Kosyakov 1954) devised a method by which M and N could be demonstrated. Their work was confirmed by Boorman and Dodd (1943). Whereas Boyd and Tayian (1935) could not detect M and N in boiled erythrocytes, Kosyakov (1954) was able to do so, and also showed these antigens to be heat stable.

Rh Groups

In 1939, Levine and Stetson reported a case of erythroblastosis fetalis and ascribed this disease of the newborn to sensitization of the mother to a blood antigen the fetus had inherited from the father. It is now known that this proposed explanation of the disease is correct and that the antigen operating in the case described was one of those now known as Rh. Levine and Stetson, however, did not propose any name for the new blood factor (on such a slender hair sometimes dangles the apple of priority). It was not until Landsteiner and Wiener (1940) discovered that serum from one of their rabbits immunized with rhesus erythrocytes detected a new factor in human blood that the term Rh was introduced. The new factor still might not have attracted any more attention than had others previously reported had not Wiener and Peters (1940) shown that certain transfusion reactions were due to sensitization to Rh and Levine et al. (1941) shown that Rh incompatibility between mother and fetus could be the cause of erythroblastosis fetalis.

Reduced to the simplest terms, the way Rh incompatibility can cause erythroblastosis fetalis is apparently this: The mother is Rh-negative, and the fetus inherits the Rh blood factor from his father.

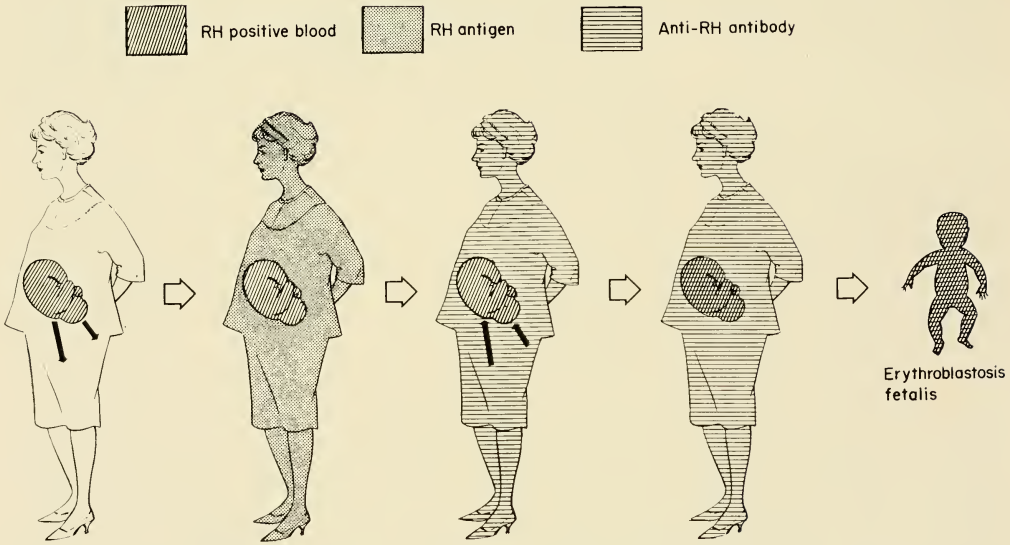

Fig. 4-2. Scheme showing mechanism of production of erythroblastosis fetalis.

Fetal antigen-containing erythrocytes, or perhaps in some cases merely antigen from disintegrating fetal erythrocytes, passes the placental barrier or crosses minute breaks in the placental capillaries and starts an immune response in the mother. This results in the appearance of anti-Rh antibodies in the maternal circulation. These diffuse through the placental barrier back into the fetal circulation (Fig. 4-2) where they combine with the fetal erythrocytes causing red cell destruction and, in severe cases, anemia, jaudice, edema, and death.

It should be mentioned that erythroblastosis fetalis, although serious when it occurs, is rare. In the United States it occurs in about one out of 400 births and rarely affects the first pregnancy, even when Rh incompatibility exists.

The Rh blood groups are much more complicated than would the case be if there were simply two possibilities: Rh-positive (the erythrocytes contain the Rh antigen) or Rh-negative individuals (the erythrocytes do not contain the Rh antigen). Our knowledge of the Rh blood groups, which extends now to five basic Rh antigens, is the result of the work of many able and industrious researchers. Only a simplified outline of it can be given here.

Although it does not have priority, the notation devised for the Rh antigens by Fisher and Race (see Race, 1944) has proven clearer and more convenient than Wiener's notations. The five basic Rh antigens are, according to Race, designated as C, c, E, e, and D. Genetically C and c are alleles, and so are E and e. It is not yet certain whether this means that three closely linked loci are involved in their mechanism of inheritance, as the British workers believed, or a series of alleles at one locus, as Wiener continues to maintain. In any case, the allelic behavior of C and c and E and e is an important phenomenon for their application in legal medicine. The allele corresponding to D is d, but no d antigen has been demonstrated with certainty.

The number of Rh blood groups that can be distinguished depends on the number of anti-Rh agglutinins available. Anti-e is seldom available and anti-c is not always easy to get for routine determinations. If all five agglutinins are at hand, three different blood groups can be distinguished with respect to the C locus, namely C-positive c-negative, C-negative c-positive, and C-positive c-positive. (Since

C and c behave like alleles, the possibility of a C-negative c-negative grouping does not exist.) Use of anti-E and anti-e further subdivides each of these three types into three others, and the use of anti-D subdivides all types once more into D-positive and D-negative. Thus, eighteen types ($3 \times 3 \times 2 = 18$) of Rh antigen can be distinguished. If anti-e is not available, as is usually the case, he number of Rh types becomes twelve ($3 \times 2 \times 2 = 12$) (Table 4-4).

Variants of these antigens, described by the British workers as further alleles, have been found, and antigens which seem to be due to other gene loci on the same chromosome are known. A rare type of blood, classified D, which contains only the D antigen, has been found.

TABLE 4-4

Reactions of the Twelve Rh Blood Groups Distinguished by Four Anti-Rh Sera

	Reaction with serum			
Group	Anti-C	Anti-D	Anti-E	Anti-c
1. cde	0	0	0	+
2. cdE	0	0	+	+
3. cDe	0	+	0	+
4. cDE	0	+	+	+
5. Cde/c	+	0	0	+
6. CdE/c	+	0	+	+
7. CDe/c	+	+	0	+
8. CDE/c	+	+	+	+
9. Cde/C	+	0	0	0
10. CdE/C	+	0	+	0
11. CDe/C	+	+	0	0
12. CDE/C	+	+	+	0

The frequencies of the occurrence of Rh antigens vary widely in different populations. The Rh-negative type (cde) is most strikingly absent from Asian and Pacific populations and the American Indians. It has its highest frequency in the Basques, a population in certain regions of France and Spain, and certain inhabitants of Switzerland. The Basques speak a non-Indo-European language and are known to represent the remnant of an earlier European population which was once dispersed over a much wider area, including perhaps North

Africa. The D antigen is most frequent in African populations and is so much more common there that it could almost be called an African antigen.

Stability of Rh Antigens

The chemical nature of the Rh antigens is still completely unknown (see, however, Chapter 7). The antigens appear to be less stable than A, B, M, and N; at least Kosyakov (1954) reported that they are destroyed by boiling for 10 minutes.

Other Blood Groups

Once laboratories were set up to examine routinely human sera which showed atypical agglutination reactions, the discovery of other human blood groups followed rapidly. It is doubtful if all have yet been reported. Those already demonstrated have generally been named after the donor in whose blood the new antigen or antibody was first identified. They have names such as Lutheran, Lewis, Duffy, Kell, and Kidd. A blood factor which may possibly be different from any of these has been found with the aid of a plant agglutinin from peanuts (see Chapter 5).

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CHAPTER 5

Plant Agglutinins (Lectins) I

Specificity of Proteins Other Than Antibodies

Antibodies are not the only large molecules with specific biological activity. Enzymes (also proteins) and hormones, many of which are proteins, also exhibit this phenomenon. Enzymes, outstanding examples in this respect, show various degrees of specificity. Some enzymes, such as barley maltase and succinic acid dehydrogenase, are very specific, catalyzing one reaction and only one reaction. Other enzymes are specific for a particular chemical grouping in their substrate. Enzymes catalyzing reactions in which optically active substances such as sugars or amino acids are involved frequently act primarily or exclusively on one of the enantiomorphs. Even enzymes such as trypsin attack only certain linkages in their substrates. Enzymes can be inhibited by an excess of one of the products of the reaction they catalyze, a behavior reminiscent of the specific inhibition of antibody-antigen reactions by haptens.

Although enzymes resemble antibodies in many ways, there are striking differences. An enzyme combines with its substrate and then catalyzes a chemical reaction in which the substrate is involved. The result is often complete destruction of the substrate. Antibodies, on the other hand, have no known catalytic activity and do not themselves cause chemical changes in the antigens with which they combine.

Other proteins exhibit specific combining power. Serum albumin has the power of binding certain dyes and a number of other natural and synthetic substances (Klotz, Walker, and Pivan, 1946; Karush, 1950).

Grabar (1947) suggests that the power of plasma proteins to combine with various substances explains one of their important roles—that of carrier (*transporteur*). According to his view, the lipid carrying role of beta globulins is analogous to the function of antibodies in their union with antigens.

Plant Agglutinins

Possession of proteins capable of such firm and relatively specific combination with other substances is not confined to the higher animals. It has long been known that extracts of certain plant seeds will bring about the agglutination of animal erythrocyte suspensions to which they are added. In fact, the agglutinative action of extracts of the castor bean, *Ricinus communis* (Table 5-1), was discovered be-

TABLE 5-1

Agglutination of Animal Erythrocytes by Ricin[a]

	Extract of *Ricinus communis*, diluted							
Red cells from	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096	1:8192
Rabbit	4	4	4	4	4	3	3	1.5
Cat	4	4	4	4	4	4	1.5	0.5
Man	4	4	4	4	1.5	0.5	0	0
Chicken	4	4	4	1.5	0	0	0	0
Rat	4	4	3	0	0	0	0	0

[a] Numbers indicate degree of agglutination; 4 indicates complete agglutination, 0.5, weak agglutination, 0, no agglutination.

fore agglutinins for erythrocytes were demonstrated in the blood of animals (Lau, 1901), and was described soon after bacterial agglutination. Extracts of other plant seeds, such as *Abrus precatorius* and certain other Leguminosae, were shown to have similar action.

Because these plant agglutinins act on the red cells of several animal species they were called nonspecific by many workers. Yet Landsteiner (1945) observed that the substances are not without a certain degree of specificity. He illustrated this fact with a little table in his book on the specificity of serological reactions (Table 5-2). It

TABLE 5-2

Titers of Different Plant Agglutinins for the Red Cells of Different Species[a]

	Titer for blood of:		
Agglutinin from:	Rabbit	Horse	Pigeon
Beans	125		2000
Lentils	160		0
Abrus precatorius		128	256
Castor beans		4	512

[a] After Landsteiner, 1945.

will be seen that some of the seed extracts show specificity, since they agglutinate the erythrocytes of one species more strongly than those of another and in one case do not agglutinate blood of a certain species at all.

Blood Group-Specific Plant Agglutinins

One day toward the end of 1945, looking at this table (Table 5-2) in the new edition of Landsteiner's book, I was seized with the idea that if such seed extracts could show species specificity, they might even show individual specificity; that is, they might possibly affect the red cells of some individuals of a species and not affect those of others of the same species. I asked one of my assistants to go out and buy dried lima beans. Why I said lima beans instead of pea beans or kidney beans I shall never know. But if we had bought practically any other kind of bean we should not have discovered anything new.

The lima beans were ground and extracted with salt solution. The extract agglutinated erythrocytes of some human individuals very strongly, but those of others only weakly if at all. It was immediately evident that the differences were correlated with blood groups (Table 5-3). The agglutinin from lima beans is almost completely specific for the blood group A antigen.

This discovery was made so easily that I was disarmed. The whole process of thinking of the experiment, obtaining the materials, and testing the idea were the events of perhaps two hours. So it is not surprising, perhaps, that I failed to realize the importance of the observation and did not immediately follow it up. I was also then in

TABLE 5-3

Test of Lima Bean Extract (December 10, 1945)

Reaction of extract with cells of group:					
A		B		O	
EW	++++	LH	±	BD	0
MF	++++			BR	0
DA	++++			SJ	0
JB	++++			ON	0
AL	++++			BA	0
WCB	++++			CTS	0

the process of writing the second edition of my book on immunology. I did include a short and rather obscure reference to the observation in the new edition (Boyd, 1947).

After about two years, I returned to the study of the plant agglutinins. In 1949, I published a report on 262 varieties of plants belonging to sixty-three families (Boyd and Reguera, 1949). Of these plants, 191 showed no agglutinating activity. Some agglutinated human erythrocytes of all blood groups. Extracts of certain varieties of *Phaseolus limensis* and *Ph. lunatus* agglutinated strongly only blood of groups A and AB. One species only, *Vitis aestivalis,* gave a weak reaction only with B, but I have not been able to reproduce this result with later material.

Meanwhile, in 1948, Renkonen had published a paper dealing with independent studies on fifty-seven species belonging to twenty-eight genera. Among the blood group-specific plants he studied were *Vicia cracca,* specific for A, and *Laburnum alpinum, Cytisus sessifolius,* and *Lotus tetragonolobus,* specific for H.

A number of laboratories are now engaged in the study of these interesting substances. Reviews have been published by Krüpe (1956), Mäkelä (1957), and Bird (1959). Seeds of a number of plants are reported to contain anti-A; that of *Dolichos biflorus* reacts so much more strongly with A_1 than with A_2 as to be virtually specific for A_1 (Bird 1951). An anti-N has been found in *Vicia graminea* (Ottsooser and Silberschmidt, 1953) and, more recently, in *Bauhinia purpurea* (Mäkelä, 1957; Boyd, Everhart, and McMaster, 1958). An anti-M is on the market.

I shall discuss the nature of the plant agglutinins in Chapter 6. In the meantime may I anticipate by saying that although it is proper to refer to them as agglutinins, as has been done for more than half a century, it would be begging the question to refer to them as antibodies. In fact, I do not believe that this is what they are. I have therefore suggested referring to these proteins as lectins, from the Latin *legere,* to pick out or choose (Boyd and Shapleigh, 1954a), in order to call attention to their specificity without implying any assumptions concerning their origin. I suggested that the term be applied also to those normal antibodies supposedly not the result of antigenic stimulus. But, if Jerne's "natural selection" theory of antibody formation proves to be correct (Chapter 2), it may turn out that the difference between "normal" and "immune" antibodies is not as great as has been thought. In that case the term lectin may come to be restricted to antibody-like plant proteins. In fact, there already seems to be a tendency to use the word in this way.

No good anti-B lectin is routinely available. Extracts of *Sophiora japonica* agglutinate blood of group B more strongly than that of group A but react too strongly with A to be satisfactory as a laboratory anti-B reagent (Krüpe, 1953). *Euonymus europeus* extracts contain anti-B and anti-H (Schmidt, 1954). *Marasmius oreades,* which sometimes furnishes a satisfactory, though weak, anti-B, is a small mushroom not commercially available (Elo, Estola, and Malmström, 1951). The best anti-B is said to be that from *Bandeiraea simplicifolia* (Mäkelä and Mäkelä, 1956), although the samples of this plant I have tested have been disappointing.

Because of the absence of an anti-B, lectins are not used routinely in the determination of the ABO blood groups, in spite of the fact that satisfactory anti-A is available from several plants. However, the anti-A_1 of *Dolichos biflorus* and the anti-H of *Ulex europeus* make an ideal combination of reagents for the routine determination of the subgroups of A and AB (Boyd and Shapleigh, 1954c), as shown in Table 5-4. Anti-A lectins, especially from lima beans, have had a number of applications in special experiments where a large amount of anti-A agglutinin is needed (Atwood and Scheinberg, 1958). Testing for H substance in saliva, by inhibition of the anti-H of Ulex (Boyd and Shapleigh, 1954b), has become the preferred method of diagnosing secretors and nonsecretors (Table 5-5).

TABLE 5-4

Determination of Subgroups of A and AB with Lectins[a]

Subgroup	Reaction with extract of:	
	Dolichos biflorus	*Ulex europeus*
A_1	++++	0
A_2	0	+++
A_1B	+++	0
A_2B	0	+++

[a] Boyd and Shapleigh, 1954c.

Lectins have been used by Morgan and Watkins (1956) to show that the blood group antigen of group AB individuals is not a mixture of A and B substances, but a unique molecule containing both A and B specificities. The anti-N of *Vicia graminea* is actually better than the absorbed anti-N prepared from immune rabbit serum and would doubtless be used routinely if more of the tiny seeds of this South American plant were available. This lectin has already proven valuable in clarifying the MN system in chimpanzees (Levine et al., 1955). The anti-N of Bauhinia is not quite as good, but may nevertheless come into routine use because the seeds are available in many parts of the world (Boyd, Everhart, and McMaster, 1958).

TABLE 5-5

Inhibition of Anti-H (Ulex) by Salivas of Secretors and Nonsecretors[a]

Blood groups	Reaction of Ulex lectin with group O cells Saliva, diluted				
	1:2	1:4	1:8	1:16	1:32
A (secretor)	0	0	0	0	0
A (nonsecretor)	++++	++++	++++	++++	++++
B (secretor)	0	0	±	+	++
O (secretor)	0	0	0	0	0
O (nonsecretor)	++++	++++	++++	++++	++++

[a] Boyd and Shapleigh, 1954b.

Another example of the practical use of lectins was discovered by Levine, Celano, Lange, and Berliner (1957). Using the anti-N of *Vicia graminea*, they observed that horse erythrocytes contain the N antigen, a fact that absorbed rabbit sera had failed to reveal because species-specific agglutinins had remained in the absorbed sera. The finding of N in the erythrocytes of the horse led Levine and his colleagues to look for natural anti-M in horse serum. Having found it, they were led to predict that the horse should be a good producer of immune anti-M. This prediction was verified, and thus a new and abundant source of immune anti-M was discovered.

Until recently all plant agglutinins tested fell into two categories: either they agglutinated the red cells of all human beings and were thus considered of little interest because they were "nonspecific," or they reacted with one of the already known agglutinogens of human blood. (It is a striking fact that in spite of the thousands of species of plants already tested, no lectin reacting specifically with any of the Rh antigens has been found. I shall suggest a possible explanation for this in Chapter 7.) Recently, Boyd et al. (1959) found in extracts of ordinary peanuts (*Arachis hypogaea*) an agglutinin which seems to be an exception to this rule. It agglutinates some human erythrocytes and does not agglutinate others. But the agglutinogen detected, if a distinct agglutinogen is involved, seems to be different from A, B, H, M, N, P, S, s, C, D, E, c, e, V, Fy^a, Fy^b, K, k, Le^a, Le^b, Lu^a, Lu^b, Jk^a, Jk^b, or Js. Unfortunately the new agglutinin is very weak and works only with erythrocytes suspended in serum albumin.

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CHAPTER 6

Plant Agglutinins (Lectins) II

Nature of Plant Agglutinins

There is no reason to suppose that the blood group-specific agglutinins for which I proposed the name lectins are essentially different from the "nonspecific" agglutinins which have been known so long. Ricin, from *Ricinus communis,* has been studied more thoroughly than any other of the plant agglutinins. Although a toxin as well as a hemagglutinin, it is, on the whole, a typical lectin. The chemistry and immunology of ricin were studied and reviewed fairly recently by Kabat, Heidelberger, and Bezer (1947) and by Krüpe (1956). It is a globulin (i.e., soluble in salt solutions but not in distilled water), with an isoelectric point of 5.4-5.5 and a molecular weight of about 80,000. It can be crystallized; the crystalline protein is highly toxic and strongly hemagglutinative.

The most complete immunochemical study on a specific lectin carried out so far seems to be that of Boyd, Shapleigh, and McMaster (1955). These authors found this anti-A lectin (from lima beans) to be globulin also. They were not able to obtain it in crystalline form but studied concentrated and partially purified preparations of which about 36 per cent were specifically reactive with A antigen. The partially purified material was electrophoretically heterogeneous but nearly homogeneous in the ultracentrifuge. The observed sedimentation constant suggested a molecular weight of about 80,000.

Boyd and co-workers observed that the lectins, in addition to their ability specifically to agglutinate erythrocytes of the appropriate blood groups, precipitate specifically with the purified antigens (Boyd

and Shapleigh, 1954a; Bird, 1959). Boyd, Shapleigh, and McMaster made a quantitative study of the precipitin reaction of their partially purified lima bean anti-A and purified A antigenic substance from hog stomach. The general course of the precipitin reaction was found to be very similar to that of the precipitation of A substance by human anti-A antibody (Fig. 6-1).

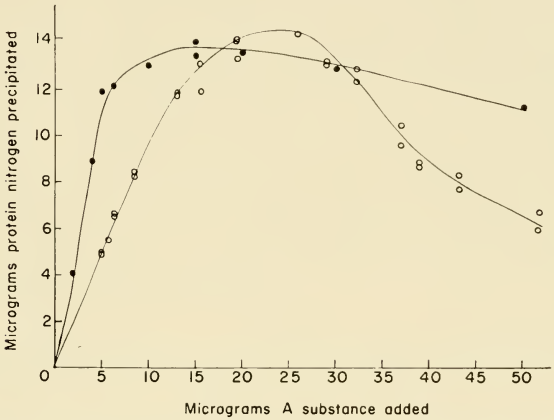

Fig. 6-1. Protein nitrogen specifically precipitated from anti-A lima bean lectin solution (open circles) by hog A substance, compared with nitrogen precipitated from human anti-A serum (solid circles).

A characteristic of the lectins, and one that strikingly differentiates them from immune antibodies, is their homogeneity. I do not mean physical homogeneity, for in most cases the lectins have not been purified sufficiently for us to know whether they are electrophoretically and ultracentrifugally homogeneous.* I mean they are homogeneous in their affinity for red cell antigens.

* By eluting proteins of different mobilities from paper electrophoresis paper, Ensgraber, Krüpe and Ensgraber-Hattingen (1960) obtained evidence suggesting that the agglutinins of twelve species studied by them were not homogeneous electrophoretically. In two cases they were able to obtain fractions of the total agglutinin present that did seem to be electrophoretically (and ultracentrifugally) homogeneous.

When an immune serum or a "normal" isoagglutinin is found to agglutinate more than one type of cell, absorption with one of these types will generally remove the antibodies which affect it, leaving the antibodies which react with other types of cells. The method of preparation of a reagent for A_1, used before the introduction of Dolichos lectin for this purpose (see Table 5-4 in Chapter 5) demonstrates the behavior of "normal" isogglutinin. The serum of an individual of group B agglutinates erythrocytes of both subgroups A_1 and A_2. Absorption with A_2, however, removes the antibody which reacts with A_2 cells, leaving an anti-A_1 agglutinin. Absorption of an antibody to hen ovalbumin with duck ovalbumin removes the duck-reactive antibody, leaving the anti-hen antibodies (some of which will react also with ovalbumins of other avian species).

If it is attempted to repeat such an experiment with a plant agglutinin instead of an antiserum from an animal, the results are generally different. Absorption with one type of cell of a lectin that agglutinates two different types of cells nearly always removes both types of antibodies. If, for example, we try to make the lima bean

TABLE 6-1

Effect of Absorption on Agglutinating Activity of 10 Per Cent Solution of Lima Bean (Sieva) Proteins[a]

	Extract, diluted										
Test cells	U[b]	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
Before absorption											
A_1	4	4	4	4	4	4	4	4	3	2	±
B	4	4	3	0	0	0	0	0	0	0	0
O	0	0	0	0	0	0	0	0	0	0	0
After 3 absorptions with B cells[c]											
A_1	4	4	4	4	4	4	3	2	1	0	0
B	±	0	0	0	0	0	0	0	0	0	0
O	0	0	0	0	0	0	0	0	0	0	0

[a] Boyd and Shapleigh, 1954d.

[b] U = undiluted.

[c] After absorption, 0.05 ml. of the indicated dilution of the protein solution was mixed with 0.05 ml. of a 1% suspension of erythrocytes of the indicated group and the mixture centrifuged, shaken, and read microscopically.

lectin more specific by treating it with B cells to remove the anti-B, we find we can do so only by absorbing an amount of the protein sufficient to reduce the lectin concentration to such a point that it will no longer agglutinate B cells (Table 6-1). This is seen from the fact that, while the absorption was successful in lowering the titer of the lectin against B cells about two stages, it also lowered the titer against A_1 cells two stages (Boyd and Shapleigh, 1954d).

Another indication of the homogeneity of lectins is the observation of Morgan and Watkins (1953): the agglutinin of *Sophora japonica* can be absorbed by either A_1 or B cells, but the agglutinin, on being eluted from either type of cell, still agglutinates A and B cells as before, showing that no separation into molecules of different specificities has been effected.

This homogeneity of the lectins possibly sheds light on what their function may be in the plant, and also, because of its contrast with antibodies, has a bearing on theories of antibody formation.

Specificity of Plant Agglutinins

We have already seen that there are degrees of specificity. The specificity of an antibody may be described as *low* if it reacts with a large number of antigens, especially if they are closely related chemically. Specificity is said to be *sharp* if an antibody reacts only with a small group of chemically closely related antigens. An antibody may be said to have absolute specificity if it reacts with only one antigen.

When blood group-specific plant agglutinins were first discovered, it was natural to suppose, since their reaction with the blood group antigens was thought to be merely a chemical accident, that their specificity was less sharp than that of the normal isoagglutinins. The contrary has proved to be true, at least in some cases.

The specificity of some lectins is very sharp. The anti-A of human group B plasma, for example, reacts nearly as well with A_2 as with A_1 cells. The lectin of *Dolichos biflorus,* on the other hand, has a much greater affinity for A_1 than for A_2 (over 500 times as great), so that it is virtually specific for A_1. Human anti-A reacts also with the Forssman antigen, the J substance present in the blood of some cattle, the R antigen present in some sheep, and hog A substance,

whereas the Dolichos lectin is not specific for any of these, but detects a previously undescribed heterogenetic factor present in the erythrocytes of sheep, goat, horse, dog, and pig (Bird, 1959).

The anti-A of lima beans is somewhat less specific; its affinity for A_2 is higher, and, when concentrated, it weakly agglutinates B cells also. The anti-H lectins are still less specific, for, when sufficiently concentrated, they may agglutinate A_1 and B cells. This, however, might be because human erythrocytes of these groups contain some H antigen.

In addition to these relatively specific lectins, others are known which seem to react with more than one receptor on the red cell, such as A and B, or B and H. Finally, we come to those which agglutinate human cells of all groups. Even these, however, may have their own specificity, as I shall mention later.

As we shall see in the next chapter, human isoagglutinins react not only with purified A and B substances but also with certain fragments into which these antigens can be split, as by hydrolysis. In the case of fragments too small to precipitate, specific activity has to be demonstrated by the inhibition technique. In view of what we know about antigenic determinants, we should expect a limit to this fragmentation process; i.e., if the A blood group antigen, for example, is split into portions that are too small, the fragments will no longer show A specificity or, in other words, will no longer inhibit the reaction of anti-A agglutinin with A antigen. It was found the smallest fragment of the A antigen which specifically inhibited the reaction of human anti-A antibody with the A antigen is a disaccharide containing *N*-acetylgalactosamine as a terminal group. (The structure of this disaccharide will be discussed in Chapter 7.) Strikingly enough, anti-A agglutinins from plants were inhibited not only by this disaccharide but by *N*-acetylgalactosamine itself (Morgan and Watkins, 1959). From this observation we could draw either of two opposed conclusions. We could say that plant anti-A reagents are less specific than human anti-A, since they are inhibited by a simpler substance, or we could say they are more specific, since they cross-react less with other portions of the A antigen.

In the case of anti-H agglutinins the plant reagents have not proven to be any less specific than those of animal origin. The anti-H of eel serum is inhibited by L-fucose, and L-fucose inhibits the anti-H of

Lotus tetragonolobus and *Ulex europeus*. L-Fucose does not inhibit the anti-H of *Cytisus sessilifolius* or of *Laburnum alpinum*, but salicin, a glucoside of D-glucose and saligenin, does (Bird, 1959). If this means that the lectins of Cytisus and Laburnum are directed toward a part of the H antigen different from that recognized by the animal anti-H reagents, it might suggest that the specificity of the lectins is greater, not less than that of the animal agglutinins.

The anti-H of *Lotus tetragonolobus* is inhibited also by 2-deoxy-L-fucose, L-galactose, 6-deoxy-L-talose, D-arabinose, and *N*-acetyl-glucosamine. Morgan and Watkins (1953) pointed out that, except for the last, all the inhibiting sugars, when written in the pyranose form, have the same configuration at carbon atoms 3 and 4 (Fig. 6-2). In all of them the hydroxyl groups are on the same side of the pyran ring and pointing down.

Krüpe (1956) noticed that the sugars which inhibited the anti-(A+B) agglutinin of *Sophora japonica* (*N*-acetyl-D-galactosamine,

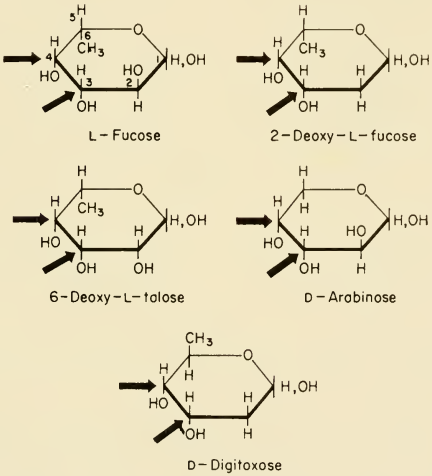

Fig. 6-2. Haworth formulas of sugars inhibiting anti-H of *Lotus tetragonolobus*. Arrows point to carbons 3 and 4, which have the same configuration in all these substances. (Redrawn from Morgan and Watkins, 1953).

D-galactose, lactose, melibiose, L-arabinose, and D-fucose) also all had the same configuration at carbons 3 and 4. Here, too, the hydroxyl groups at carbons 3 and 4 are at the same side of the ring, but are pointing up, which is just the opposite of that found in the sugars inhibiting Lotus (Fig. 6-3).

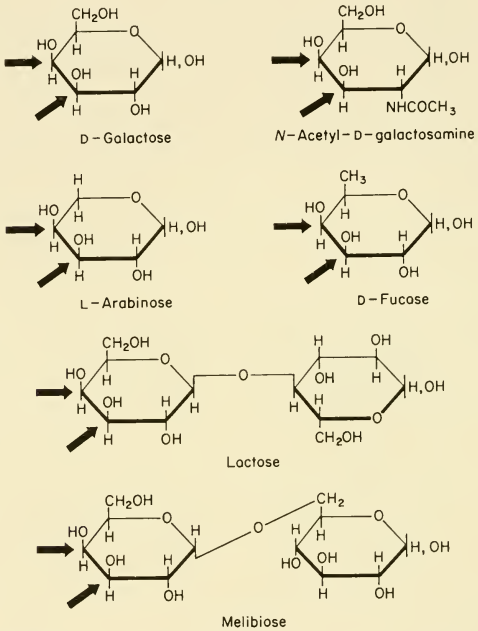

Fig. 6-3. Haworth formulas of sugars inhibiting the anti-(A+B) of *Sophora japonica*. (Redrawn from Krüpe, 1956).

Mäkelä (1957), who made a much more extensive study of plant agglutinins and their inhibition reactions, suggested that monosaccharides fall into four classes with respect to their specific inhibiting activity for plant agglutinins and that this is based on their configuration at carbons 3 and 4 (Fig. 6-4).

The assignment of the aldohexoses and aldopentoses to Mäkelä's four groups and the steric similarities of these sugars in each classification are shown in Fig. 6-5. The relation between the pentoses and hexoses shown in books on organic chemistry is based on possible synthetic pathways in the laboratory and does not always show the actual spatial relations of the ring structures.

Krüpe observed that the agglutinin of *Ricinus communis* was inhibited by sugars which fall into Mäkelä's group 2, and the agglutinin of *Pisum sativum* by sugars of group 3. Apparently these two "nonspecific" agglutinins do show a certain degree of specificity. In his

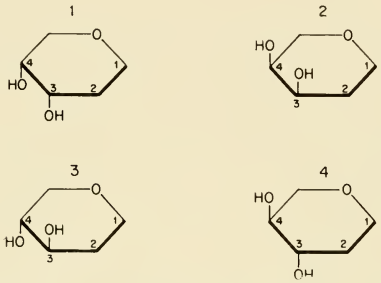

Fig. 6-4. Classification of pyranose forms of sugars into four groups on the basis of the configuration of carbons 3 and 4 (Mäkelä, 1957).

more extensive study Mäkelä found many other leguminous seeds having agglutinins which fell into one of these two classes. Other legumes did not fall into either class. In addition to seeds with agglutinins inhibited by sugars of groups 2 and 3, Mäkelä's tables give examples of seeds not inhibited by any sugars tested but inhibited by blood group substances and some seeds that are not inhibited by any of the substances tried.

Among plants the seeds of which contain agglutinins inhibited by sugars of group 2 are *Bandeiraea simplicifolia,* various species of Bauhinia, *Sophora japonica,* various species of Crotalaria, various species of Cytisus, various species of Caragana, *Wisteria chinensis, Coronilla varia,* various species of Erythrina, and *Glycine soja.* Inhibited by sugars of group 3 are *Parkia filicoidea, Lathyrus latifolius,*

Lens culinaris, Pisum sativum, and various species of Vicia (Table 6-2).

Fig. 6-5. Steric relations of aldopentoses and aldohexoses (Boyd, 1960).

TABLE 6-2

Lectins[a] Inhibited by Sugars of Group 2 and 3.

Group 2	Group 3
Bandeiraea simplicifolia	*Parkia filicoidea*
Bauhinia spp.	*Lathyrus latifolius*
Sophora japonica	*Lens culinaris*
Crotolaria spp.	*Pisum sativum*
Cytisus spp.	Vicia spp.
Caragana spp.	
Wisteria chinensis	
Coronilla varia	
Erythrina spp.	
Glycine soja	

[a] Extracted from plant seeds of the species listed.

It seems clear that these plant agglutinins are not nonspecific, but react with a definite chemical structure in the red cell, probably one having as terminal group a sugar of group 2 or 3, as the case may be. It happens that the erythrocytes of all human beings contain both these particular receptors; so therefore no individual differences are found in the reactions of these lectins with human erythrocytes. Further study with more complicated carbohydrates will enable us to make a better guess at the detailed structure of these receptors. As to the receptors with which other plant agglutinins combine we have as yet no clue.

In a systematic study of the inhibition of two "non-specific" lectins, that of *Ricinus communis* and that of *Bauhinia purpurea* (dialyzed free of the group 2 sugars making it *N*-specific), Boyd and Waszczenko-Zacharczenko (1961) found considerable similarities, but some differences. *Bauhinia* lectin was inhibited by sugars of Mäkelä's group 3, but Ricinus was not. It was concluded that the receptors in the human erythrocyte with which these two lectins combine, though similar, are not identical. Both lectins were inhibited by "unnatural" sugars of group 4.

In some cases the addition of an inhibiting sugar to a non-specific plant agglutinin does not suppress all activity but leaves the preparation able to agglutinate cells of certain blood groups, thus revealing a new specificity. Mäkelä (1957) found that the agglutinin of *Bandeiraea*

simplicifolia became B-specific when tested against cells suspended in 2 per cent glucose. A suitable concentration of galactose makes the anti-(A+B) agglutinin of *Calpurina aurea* A-specific (Bird, 1959). Boyd, Everhart, and McMaster (1958) found that some preparations of *Bauhinia purpurea* were nonspecific (really specific for sugars belonging to group 2), but could be made N-specific by the addition of D-galactose or other sugars of group 2. Most nonspecific plant agglutinins, however, do not develop a new specificity when treated with inhibiting sugars in this way. The three lectins just mentioned would seem to be exceptional in this respect.

Role of Agglutinins in the Plant

We do not really know the role in the plant of the proteins which we recognize by their ability to agglutinate certain types of erythrocytes. In speculating about this role we may follow several lines of thought.

One possible approach, but in my opinion a naive one, is to assume that because the lectins behave like antibodies they are real antibodies. There are several arguments against this assumption: (a) Although the literature on plant immunity is enormous, it has not been demonstrated that plants manufacture antibodies. (b) There is no evidence that the plants have ever been exposed to the blood antigens with which their lectins react. It is extremely unlikely, for example, that *Vicia graminea* has ever come in contact with the blood group N antigen. (c) Lectins may occur in some varieties of a species and be absent in others. This difference persists even when the varieties are grown in identical environments; experiments carried out in Puerto Rico have indicated that the difference is hereditary (Schertz, Jurgelsky, and Boyd, 1960).

Another point of view assumes that the configuration which enables the plant proteins to combine specificially with certain blood group antigens is merely an accidental feature of their structure and that the proteins are present in the seed merely as storage material, or for some similar purpose.

A third point of view, which I favor, holds that it is no accident that the lectins are adapted to combine specifically with certain carbohydrates but that their function in the plant is to combine with,

transport, and perhaps immobilize in the seed one or more of the carbohydrates with which they have the power to combine. Krüpe (1956) first suggested the possible role of the lectins as "Kohlenhydratfixierer" ("carbohydrate catchers"), and Boyd, Everhart, and McMaster (1958) also thought that lectins might so function in the plant.

Lessons from the Study of Lectins

The study of plant agglutinins promises to throw new light on the specificity of the blood group antigens and on the nature and number of carbohydrate groupings which are present on the surface of the erythrocyte. I shall discuss this in the following chapter. Study of inhibition reactions of the lectins has already thrown considerable light on the structure of the ABH antigens. The difference in specificity between lectins and human and animal agglutinins, whether we care to regard this difference as evidence that lectins are less specific or more specific, makes lectins particularly suitable for a study by the inhibition reaction of the cell receptors. (See Chapter 7.)

The greater homogeneity of the lectins with regard to specificity presents an interesting contrast with antibodies and might suggest that a protein molecule which has a certain specificity as a part of its role in metabolism is likely to be more uniform in this respect than a gamma globulin mixture which has acquired a certain specificity by some process of natural selection. This line of thinking may support the antibody-formation theory of Jerne and subsequent modifications thereof.

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CHAPTER 7

Blood Group Antigens

Sources of Antigens for Study

The human erythrocyte is a complicated structure, the blood group antigens apparently making up only a small part of its mass. It is not surprising, therefore, that attempts to determine the structure of the blood group antigens by analyzing material isolated from erythrocytes have never given information of much value. Not only is the starting material complex and the desired antigens only a small portion of it, but the antigens seem to be bound in some way to the lipids, and possibly to the proteins, which are present on the surface of the red cell, making purification extremely difficult (Morgan and Watkins, 1959). If it were not for the much more abundant occurrence of the blood group substances, in water-soluble form, in the saliva, gastric juice, ovarian cyst fluid, and meconium of secretors, and the occurrence of closely related antigens in hog and horse stomach, little would be known today of the chemistry of blood group substances.

A number of methods of isolating and purifying blood group substances from the sources just mentioned have been described. The extraction with cold 90 per cent phenol, employed by Morgan and King (1943), used more than any other method, eliminates most of the accompanying nonspecific protein and other impurities. High-speed centrifugation and further fractionation from water and other solvents results in further purification.

Blood Group Substances A, B, H, and Le^a.

As a result of such methods, four blood group substances have been obtained in amounts sufficient for chemical study: A, B, H, and Le^a.

The Le^a antigen (one of the antigens of the Lewis blood group system) has been studied nearly as thoroughly as the ABH antigens because it also occurs in water-soluble form in body fluids.

Analytical Results

The results of chemical analyses of the ABH and Le^a blood group antigens have been disappointing: they have not revealed any chemical differences that can be correlated with differences in blood group activity. At a glance, the four antigens seem very much alike. They each contain the same two sugar components, L-fucose and D-galactose, and the same amino sugar components, D-glucosamine and D-galactosamine. They also contain the same eleven amino acids (Kabat, 1956; Morgan and Watkins, 1959). The role of the amino acids is not clear, for the specificity of the antigens seems to be determined mainly by the carbohydrate portions. Morgan believes, however, that the blood group antigens are not merely a loose combination of a macromolecular polysaccharide with protein but consist of carbohydrate chains and peptide units bound together by primary chemical bonds.

Typical analytical values for preparations of the specific substances are shown in Table 7-1. The observed differences are within the range of variation found with different preparations of the same antigen (Morgan and Watkins, 1959).

From such data we are forced to conclude that the specific serological differences between the A, B, and H antigens are due not to differences in over-all composition but to variations in the arrange-

TABLE 7-1

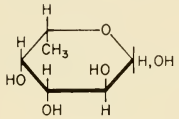
Typical Analytic Values for Preparations of Human Blood Group Antigenic Substances[a]

Substance	N, %	Fucose, %	Acetyl, %	Hexosamine, %	Reduction, %
A	5.4	19	9.0	29	54
H	5.3	18	8.6	28	50
Le^a	5.0	14	9.9	32	56
B	5.6	16	7.0	24	52
AB	5.6	17	—	26	54

[a] Morgan and Watkins, 1959.

ment of the component parts. In fact, recent evidence suggests that only certain parts of the complex polysaccharide molecules are responsible for the specific serological properties.

Of all the sugars present in the H blood group substance, for example, only L-fucose (Fig. 7-1) specifically inhibited the agglutinat-

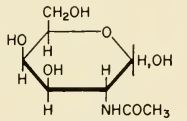


L-Fucose

Fig. 7-1.

ing action of an anti-H from eel serum. Similar results were obtained with an anti-H of plant origin, of the seeds of *Lotus tetragonolobus*. It was also found that anti-H from either of these two sources was inhibited more strongly by α-methyl-L-fucopyranoside than by the β-furanoside or by fucose alone. These results suggested that L-fucose is an important part of the H substance molecule; by analogy with Landsteiner's findings with composite haptens (p. 40), L-fucose is probably the terminal group of the specific part. The fact that the α-methylfucopyranoside inhibited better than the β-methylpyranoside suggested that the fucose was connected by an alpha linkage to the next residue of the reactive portion of the H molecule.

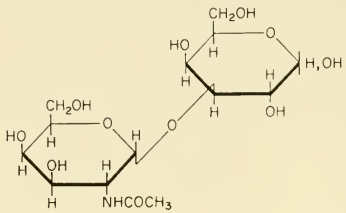
The first information concerning the role of a particular sugar in the specificity of the A substance was obtained by tests on anti-A reagents of plant origin (Morgan and Watkins, 1959). Anti-A lectins were specifically inhibited by *N*-acetylgalactosamine (Fig. 7-2). Most of the human anti-A reagents tested were not inhibited by this amino sugar but were inhibited by the disaccharide *O*-α-*N*-acetyl-D-



N-Acetyl-D-galactosamine

Fig. 7-2.

galactosylaminoyl-(1→3)-D-galactose (Fig. 7-3). This suggests that this disaccharide must be very similar to, or possibly identical with, the terminal disaccharide portion of the specific part of the human A substance. (Morgan and Watkins, 1959).



o-α-*N*-Acetyl-D-galactosylaminoyl-(1→3)-D-galactose

Fig. 7-3.

Kabat and co-workers (1956), also using the inhibition technique, found evidence bearing on the structure of the specific part of the B antigen. Of the monosaccharides present in the molecule, D-galactose was the best inhibitor of anti-B antibodies, but the galactose-containing disaccharide melibiose, the trisaccharide raffinose, and the tetrasaccharide stachyose (Fig. 7-4) inhibited even better than galactose alone (Fig. 7-5). This would have been expected if the specific part of the B antigen consisted of a terminal nonreducing galactose unit joined by an alpha linkage to another sugar unit. That the linkage is alpha is pretty well shown by the fact that α-methylgalactoside inhibits better than galactose, but the β-galactoside inhibits not as well (Fig. 7-5).

Kabat was also able to draw some conclusions about the sugar residue next to galactose in the specific side chain of the B antigen. It could not be glucose, for glucose is not a part of the B molecule. It was not likely to be another galactose, for, if it were, stachyose, which contains a terminal galactose bound by a 1→6 alpha linkage to another galactose, would be a better inhibitor than melibiose or raffinose, where the sugar next to galactose is glucose. But stachyose is no better an inhibitor than melibiose or raffinose. According to Kabat, *N*-acetylglucosamine is the only remaining possibility for the next-to-terminal sugar in the specific side chain of B antigen.

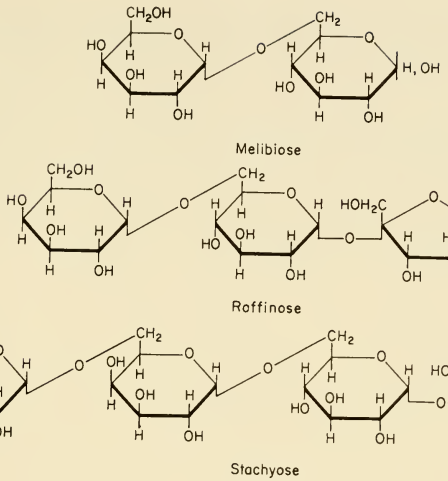


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Fig. 7-4.

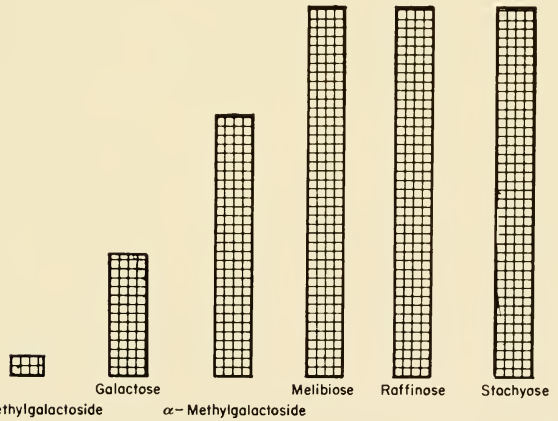
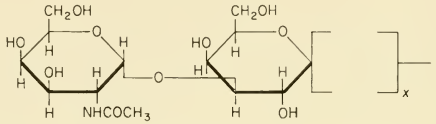


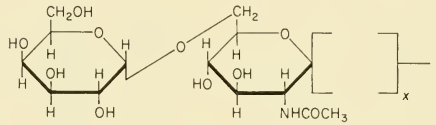
Fig. 7-5. Relative inhibiting power for anti-B of various sugars and glycosides (redrawn from Kabat, 1956).

Some of this information may eventually see practical application, for Kabat suggests that the introduction of a number of melibiosyl residues into a polysaccharide would endow it with substantial blood group B activity.

How many other sugars are present in the active side chains of the A and B molecules is not known, but on the basis of his determinations of the size of the reactive group of dextran (p. 48) Kabat believes that the total is of the order of six. This would mean that in the light of our present knowledge the specific portions of the A and B molecules would resemble the structures shown in Fig. 7-6, where x stands for a number of the order of four.



A Substance



B Substance

x = approx. 4

Fig. 7-6. Suggested structures of reactive groups of blood group A and B substances.

Additional and independent evidence for the part played by L-fucose, *N*-acetylgalactosamine, and D-galactose in H, A, and B specificity, respectively, was obtained by Watkins and Morgan (1955) from the results of enzyme inhibition by these sugars. It is known that an enzyme can be inhibited by an excess of one of the products of its action on its substrate. An enzyme preparation was available from *Trichomonas foetus* which destroyed the substrates consisting

of A, B, and H substances. As expected, the destructive action of the enzyme preparation on A substance was inhibited by *N*-acetylgalactosamine, the action on B substance by galactose, and the action on H substance by fucose.

There is at present no clue to the identity of the monosaccharide unit next to fucose in the specific part of the H substance. Our best picture of its structure is shown in Fig. 7-7, where x stands for a number of the order of five.

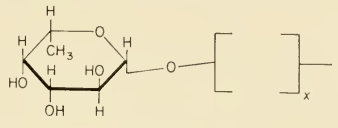


Fig. 7-7. Suggested structure of blood group H substance.

Watkins and Morgan (1957) found that the destruction of the serological activity of the Le^a antigen by the Trichomonas enzymes was inhibited by L-fucose, which suggested a role for this sugar in the specificity of the Le^a antigen. However, the agglutination of Le(a+)

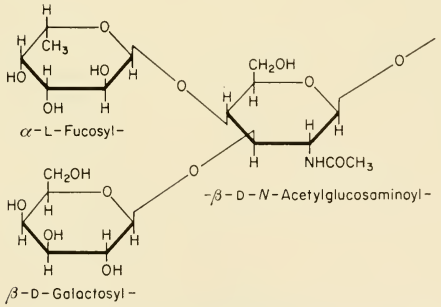


Fig. 7-8. Suggested structure of terminal portion of blood group Le^a substance (Morgan and Watkins, 1959).

red cells by human or rabbit anti-Lewis sera was not detectably inhibited by L-fucose or by any other components of the blood group substances. Certain oligosaccharides containing fucose did inhibit, however, which showed that α-L-fucopyranosyl groupings were involved in Le^a specificity (Morgan and Watkins, 1959). From a study of the inhibitory activity of various oligosaccharides, mostly isolated by Kuhn and his colleagues from human milk (Kuhn, 1957), Morgan and Watkins suggest that the terminal portion of the specific part of the Le^a substance is a trisaccharide of the structure shown in Fig. 7-8.

Action of Genes

The way in which the ABO, secretor, and Lewis genes cooperate to produce the various blood group substances found in the body fluids of persons of different genotypes is still to be worked out. Watkins and Morgan (1959) have proposed the following scheme as a first approximation. Three independent gene systems, L′ and l′, S′ and s′, and the ABO genes, are supposedly involved. In various

TABLE 7-2

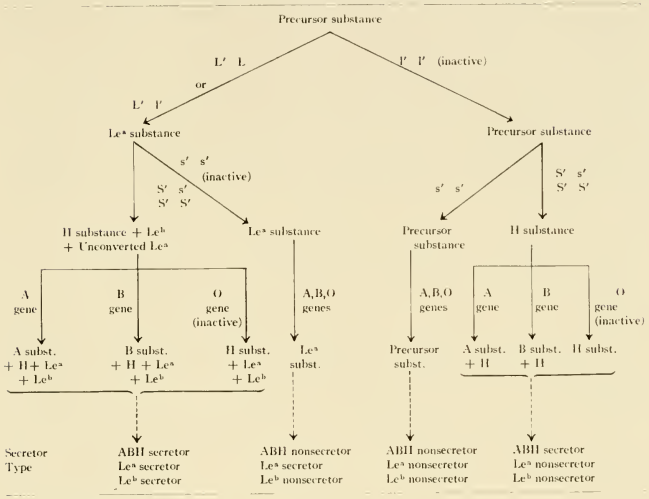
Possible Genetic Pathways for the Production of Blood Group Substances. [a]

Sequence and products of gene action			Secretor type
Precursor substance	l′ gene (inactive) →	Precursor substance (not acted on by S′, A, or B genes)	Nonsecretor ABH Nonsecretor Le^a
↓ L′ gene (+ α-fucosyl units)			
Le^a substance	s′ gene (inactive) →	Le^a substance (not acted on by A or B genes)	Nonsecretor ABH Secretor Le^a
↓ S′ gene (+ α-fucosyl units)	O gene (inactive) →	H substance + Unconverted Le^a + Le^b	
H substance + Unconverted Le^a + Le^b	B gene (+ α-galactosyl units) →	B substance + Unconverted H and Le^a + Le^b	ABH secretor
	A gene (+ α-*N*-acetyl galactosaminoyl uints) →	A substance − Unconverted H and Le^a + Le^b	

[a] Watkins and Morgan, 1959.

TABLE 7-3

Possible Genetic Pathways for the Production of Blood Group Substances, H[a]



[a] After Watkins and Morgan, 1959.

ways they act to modify the precursor substance, a mucopolysaccharide which is believed to be identical with the material found in the secretions of the individuals who secrete neither A, B, or H nor Le^a or Le^b substances. The L′ gene acts to add α-fucosyl units to this precursor substance, and the S′ gene adds still more. The B gene adds α-galactosyl units, and the A gene adds α-galactosaminoyl units (see Table 7-2).

The scheme of Table 7-2 is inadequate in some respects, and Watkins and Morgan suggest replacing it by the more complicated system shown in Table 7-3.

Other Human Red Cell Receptors

Bauhinia Receptor

In addition to the red cell receptors characteristic of the various blood groups, there are a number of receptors, some common to all

human erythrocytes, about which a certain amount of information has been gained by a study of the inhibition of lectins by carbohydrates. Let us first consider a receptor detected by extracts of *Bauhinia purpurea* (Boyd, Everhart, and McMaster, 1958). As already mentioned, extracts of the seeds of this plant can be specific for the N antigen; if they are not N-specific, they can be made so by adding galactose or disaccharide containing galactose. The anti-N specificity of Bauhinia extracts depends on the presence of sufficient amounts of one or more sugars, probably galactose or galactose

TABLE 7-4

Inhibition of Dialyzed Bauhinia Extract by Carbohydrates[a]

		Sugar, diluted								
Sugars	Cells	U	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Melibiose	M	0	0	0	0	0	0	0	0	½
	MN	0	3	3	3	3	3	4	4	4
	N	0	4	4	4	4	4	4	4	4
Raffinose	M	0	0	0	0	0	0	0	½	½
	MN	0	2	3	3	3	3	3	4	4
	N	2	4	4	4	4	4	4	4	4
Lactose	M	0	0	0	0	0	0	0	3	4
	MN	0	0	0	0	1	3	3	3	4
	N	0	0	0	2	4	4	4	4	4
	M	0	0	0	0	1	3	3	3	3
L-Arabinose	MN	1	3	3	3	3	3	3	3	3
	N	3	3	3	3	3	4	4	4	4
D-Galactosamine HCl	M	0	0	0	3	3	3	4	4	4
(neutralized)	MN	0	3	3	3	3	3	4	4	4
	N	0	3	3	3	3	4	4	4	4
D-Galactose	M	0	0	0	0	0	0	3	3	3
	MN	0	0	0	0	1	2	3	4	4
	N	0	½	1	2	3	3	4	4	4
Stachyose	M	0	0	0	½	2	3	3	3	3
	MN	0	0	4	4	4	4	4	4	4
	N	0	0	4	4	4	4	4	4	4

[a] Equal amounts of the carbohydrate solution, lectin, and cell suspension were mixed. The symbol U means that the carbohydrate solution (0.1*M*) was used undiluted. The numbers signify strength of agglutination, 4 being the strongest (all the erythrocytes stuck together in one large clump). Negative reactions are recorded as 0.

derivatives. Removal of these sugars by dialysis makes *Bauhinia purpurea* extracts nonspecific; that is, they then agglutinate human blood irrespective of blood group. The effect of certain sugars on such nonspecific Bauhinia extracts is shown in Table 7-4.

It will be seen that the nonspecific activity of Bauhinia extracts is inhibited by sugars of Mäkelä's group 2. It may even be that the red cell receptor detected by nonspecific Bauhinia extracts is the same as that detected by other plant agglutinins which are inhibited by group 2 sugars. No adequate comparison has yet been made.* From the inhibiting power of the disaccharide, trisaccharide, and tetrasaccharide containing galactose, it can be assumed that the Bauhinia receptor is an oligosaccharide containing at least one more unit beyond galactose. It probably contains several monosaccharide units, although, if it does, one can conclude that the next-to-terminal unit is not galactose, as in stachyose, for this sugar is not a very good inhibitor here. Although the Bauhinia receptor has some features in common with the B receptor, it is obviously not identical with it since it occurs in all human erythrocytes. As a matter of fact, B substance does not react with the Bauhinia agglutinin.

Peanut Receptor

Another receptor has been detected with the aid of extracts of ordinary peanuts. This plant agglutinin is also inhibited by sugars of group 2 (Table 7-5). That galactose is the most effective monosaccharide inhibitor suggests that galactose is the terminal group of this receptor also. The receptor consists of more than one sugar unit, however, since two disaccharides, trehalose and lactose (the former not even containing galactose), inhibit better than galactose. Two other disaccharides containing only glucose (maltose and cellobiose) also inhibit well. The inhibitory power of the three all-glucose disaccharides and the fact that lactose contains glucose suggest that glucose may be the next-to-terminal group in the peanut receptor. This is supported by the observation that glucose itself has some inhibiting power. The effectiveness of trehalose suggests

* In my laboratory we have recently made a detailed comparison of two lectins that are inhibited by group 2 sugars (those from *Bauhinia purpurea* and *Ricinus communis*), and obtained evidence that the two receptors are not identical (Boyd and Waszczenko-Zacharczenko, 1961).

TABLE 7-5

Inhibition of Peanut Lectin (Anti-Gy) by Sugars[a]

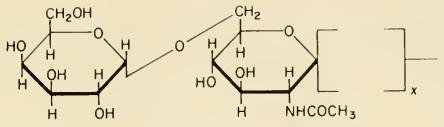
	Sugar, diluted							
	Undil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Saline (control)	++	++	++	++	++	++	++	++
D-Galactose	0	0	0	0	++	++	++	++
L-Arabinose	0	0	+	+	++	++	++	++
D-Arabinose	++	++	++	++	++	++	++	++
D-Glucose	0	+	+	++	++	++	++	++
D-Mannose	++	++	++	++	++	++	++	++
Cellobiose	0	0	0	0	0	±	+	++
Maltose	0	0	0	0	++	++	++	++
Trehalose	0	0	0	0	+	+	++	++
Melibiose	0	0	0	0	±	±	++	++
Lactose	0	0	0	0	0	0	0	0

[a] + = Agglutination of test cells, 0 = no agglutination.

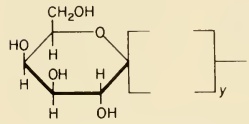
that the peanut receptor contains glucose as the next-to-terminal unit, possibly linked to the terminal galactose by a 1→1 link (the linkage in trehalose). The peanut agglutinin, in spite of a presumed galactose terminal unit, is not inhibited by B substance and in that respect resembles the Bauhinia agglutinin. The two receptors are different, however, since the peanut receptor, unlike the Bauhinia receptor, is not found on all human erythrocytes. Another sign of difference is that the peanut agglutinin is inhibited by sugars (cellobiose, trehalose, melezitose, and maltose) which do not inhibit the Bauhinia agglutinin.

There thus seem to be at least three receptors containing galactose as a terminal unit, one of them present on all human red cells, the others only on those of certain individuals. Their structure in the light of our present scanty information is shown in Fig. 7-9.

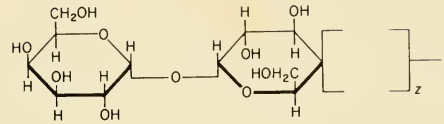
It has already been mentioned that plant agglutinins inhibited by sugars of Mälekä's group 3 react with a red cell receptor present on all human red cells. It would seem likely that the terminal unit in this receptor is a sugar of group 3. Since Mäkelä found mannose the best inhibitor for such agglutinins, one could hazard the guess that



B Substance



Bauhinia receptor



Peanut receptor (Gy)

Fig. 7-9. Suggested structure of the terminal portions of three receptors of the human red cell which contain galactose as a terminal monosaccharide unit (Boyd, 1960).

the terminal unit is mannose. We cannot go further than this on the information now available.

The RH Receptors

In my laboratory we recently applied the specific inhibition technique to a study of the Rh blood group receptors. Hackel, Smolker, and Fenske (1958) reported that anti-Rh sera are inhibited specifically by a number of ribonucleic acid derivatives, which suggested that the Rh antigens are at least partly ribonucleotide in nature. We found that human anti-D serum is also inhibited, weakly it is true, but apparently specifically, by the "unnatural" sugars of Mäkelä's group 4, including L-mannose, L-glucose, and D-gulose (Table 7-6). It is not inhibited by the "natural" enantiomers of these sugars or by any other sugars we have tried.

TABLE 7-6

Inhibition of Anti-D by 0.2*M* Solutions of Various Sugars[a]

	Serum, diluted					
Substance added	Undiluted	1:2	1:4	1:8	1:16	1:32
Saline (control)	3	3.5	4	4	3	2
D-Glucose (3)	4	3	3.5	3.5	1.5	1
L-Glucose (4)	3	2.5	0	0	0	0
D-Mannose (3)	3.5	3	3	3.5	2.5	0
L-Mannose (4)	2.5	2	0	0	0	0
D-Gulose (4)	0	1	0	0	0	0

[a] Numbers indicate strength of agglutination, from 4 = complete agglutination, to 0 = no agglutination. The numbers in parentheses after the names of sugars indicate the group of the sugar in Mäkelä's (1957) classification (see Figs. 6-4 and 6-5).

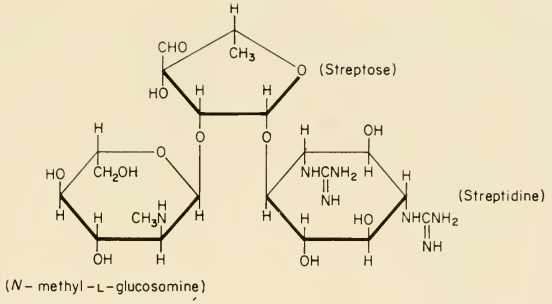
The results suggest that the D receptor may contain a sugar of group 4 as terminal unit. They are supported by the observation that streptomycin, a natural glycoside of *N*-methyl-L-glucosamine, and rutinose [6-*O*-(β-L-rhamnosyl)-D-glucose] also inhibit (Table 7-7). Streptomycin does not inhibit much better than L-mannose or

TABLE 7-7

Inhibition of Anti-D by Glycosides

	Serum, diluted					
Substance	Undil.	1:2	1:4	1:8	1:16	1:32
Saline	4	4	4	3	2	0
Rutinose (4)	4	0	0	0	0	0
Streptomycin (4)	0	0	0	0	0	0

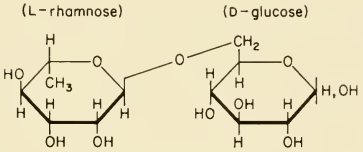
L-glucose. This is not surprising considering that the next-to-terminal unit is 5-deoxy-3-formyl-L-lyxose, which one would not expect to find in red cells (Fig. 7-10), though such preconceived notions may be dangerous. Rutinose, however, on a molar basis (the solution available was only 0.14 as strong as the other sugar solutions studied), inhibits better than streptomycin or L-mannose, which might suggest



Streptomycin

Fig. 7-10.

that the next-to-terminal unit in the D receptor is D-glucose or a similar sugar. The likelihood that the terminal unit of rutinose, L-rhamnose (Fig. 7-11), is the terminal unit of the D receptor is diminished by the observation that rhamnose itself does not inhibit.



Rutinose

Fig. 7-11.

It is hardly necessary to mention that a knowledge of the chemical structure of the D antigen could have considerable practical value. It might enable us to make good anti-D agglutinins by immunizing animals, which is now impossible. Injections of a nontoxic oligosaccharide with high D activity into pregnant women might possibly neutralize the anti-D of the maternal and fetal circulations and prevent erythroblastosis in the infant.

The inhibition behavior of anti-C and anti-E seems to be more complicated (Table 7-8). L-Glucose has some inhibitory effect on

TABLE 7-8

Inhibition of Anti-Rh Sera by D- and L-Glucose

Serum	Sugar	Serum, diluted					
		Undil.	1:2	1:4	1:8	1:16	1:32
Anti-D	Saline	3	3.5	4	4	3	2
	D-Glucose	4	3	3.5	3.5	1.5	1
	L-Glucose	2.5	2.5	0	0	0	0
Anti-C	Saline	3	3.5	3.5	3	2	1.5
	D-Glucose	3	3.5	3	3	2	1.5
	L-Glucose	3	3	0	0	0	0
Anti-E	Saline	4	4	4	3	1.5	0
	D-Glucose	3.5	0.5	0	0	0	0
	L-Glucose	3	1.5	0	0	0	0

both agglutinins, but anti-E is also inhibited by D-glucose. Other sugars of group 4 do not seem to inhibit anti-C; therefore I have no confidence yet that the terminal unit is a sugar of this group (Table 7-9). It seems possible that a sugar of group 3 is the terminal unit

TABLE 7-9

Inhibition of Anti-C

Substance	Serum, diluted					
	Undil.	1:2	1:4	1:8	1:16	1:32
Saline	4	4	4	3	3	2
D-Glucose (3)	4	4	3.5	3	4	3
L-Glucose (4)	3	3	0	0	0	0
D-Idose (4)	4	3	4	3.5	±	0
Rutinose (4)	4	3	3.5	0	0	0
Streptomycin (4)	3	3	2.5	2.5	0	0

of the E receptor, considering the effectiveness of sugars of this group in inhibiting anti-E (Table 7-10). The inhibition by L-allose, a group 2 sugar, is unexpected and is not paralleled by inhibition by other group sugars.

If it should prove that the D receptor, and possibly the C and E

TABLE 7-10

Inhibition of Anti-E

Substance	Serum, diluted					
	Undil.	1:2	1:4	1:8	1:16	1:32
Saline	3	3.5	3	1.5	2	0
D-Mannose (3)	0	0	2	0	0	0
D-Glucose (3)	0.5	0	0	0	0	0
L-Mannose (4)	2	±	0.5	0	0	0
L-Glucose (4)	3	1.5	0	0	0	0
D-Gulose (4)	2	3	1.5	0	0	0
Rutinose (4)	4	4	2	0	0	0
Streptomycin (4)	3.5	3.5	1.5	1.5	0	0
L-Allose (2)	0	±	0	0	0	0
D-Galactose (2)	3.5	4	4	3.5	2	0
L-Arabinose (2)	3	4	4	3.5	3.5	0

receptors as well, contains a sugar of group 4, it may surprise some people, for sugars of this group have not previously been found in human tissues. We may still expect many surprises regarding the natural occurrence of sugars. In the field of protein chemistry it is commonly assumed that only one enantiomer of each amino acid occurs in nature, and yet Oncley (1959) has pointed out reasons for doubting this. The finding of a derivative of L-glucose in streptomycin has already shown that some of these "unnatural" sugars occur in nature. In the next chapter it will be seen that such sugars play a role in the antigens of Salmonella and certain parasites.

Since the above was written, Dodd, Bigley, and Geyer (1960), starting from the observation that the receptor-destroying mumps virus liberates from human erythrocytes a specific anti-D inhibitor, found that *N*-acetyl-neuraminic acid and other compounds related to sialic acid inhibited anti-D but not anti-C or anti-E, thus providing a clue to the chemical differences between D and the other Rh antigens. In my laboratory we found that colominic acid, thought to be a polymer of *N*-acetyl neuraminic acid, also inhibits anti-D specifically, and suggested it might even have clinical application in preventing erythroblastosis fetalis (Boyd and Reeves, 1961).

We have also found other substances, including some amino acids, not closely related to any of the substances discussed above, to have

considerable inhibitory power. None of these compounds inhibited anti-A, anti-B, anti-H, anti-M, or anti-N. It is clearly premature to present any detailed picture of the structure of any of the Rh antigens, but if progress continues at the present rate something should be known in a few years at the latest.

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CHAPTER 8

Salmonella Antigens

Progress in bacteriology was greatly aided by the development of methods of staining microorganisms to facilitate their microscopic observation. The staining methods were developed empirically. Although their mechanism is still obscure, two of them are now known to detect fundamental and significant differences in the cellular structure of bacteria. These two reactions are the Gram stain and the acid-fast stain. On the basis of their behavior toward the reagents used in the two reactions, all bacterial species may be classified into three broad groups: Gram-positive, Gram-negative, and acid-fast. There also exist intermediate forms with poorly defined staining reactions (Dubos, 1952).

Endotoxins

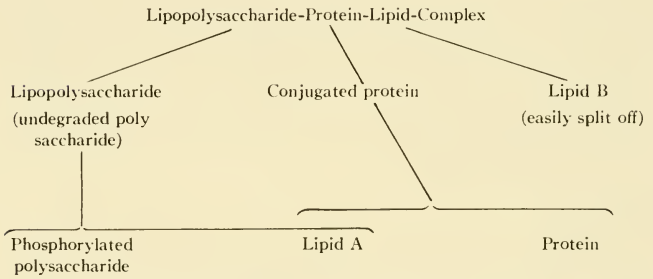
Gram-negative bacteria are characterized by the fact that, when they are dyed with a basic triphenylmethane dye such as gentian violet, the color can be removed by washing with alcohol. Gram-negative bacteria have a number of other features in common. One of the most interesting of these features to the immunologist is their content of *endotoxins*. These characteristic substances, not released to any great extent into the culture medium as the organism grows (in contrast to *exotoxins* such as diphtheria toxin), can be obtained by lysing the bacteria or by extracting them with trichloracetic acid, diethylene glycol, etc. They are toxic in animals in very small amounts (of the order of 0.001 microgram per kilogram of body weight), producing fever, leukopenia, etc.

The first important step in the study of endotoxins was the development by Boivin (Boivin and Mesrobeanu, 1933) of a method of extracting them with trichloracetic acid. Boivin reported that the endotoxins of a number of gram-negative bacteria consisted mainly of polysaccharide and lipid.

Modern knowledge of the chemistry of endotoxins derives mainly from the work of Morgan (1937, 1940, 1941, 1942) and Goebel (1945), who showed that endotoxins are complexes containing phosphorylated polysaccharide and protein. Further work by these and other workers on the degradation products of the endotoxins revealed that they have the make-up shown in Table 8-1 (Westphal and Lüderitz, 1954).

TABLE 8-1

The Endotoxin Complex of the Cell Wall of Gram-Negative Bacteria[a]



[a] Westphal and Lüderitz, 1954.

It appears that the lipid A component is responsible for many of the toxic effects of these complex substances (Schmidt, Eichenberger, and Westphal, 1958; Westphal, 1960). All the preparations of this component examined from various enterobacteria seem to be similar or perhaps identical (Westphal, 1960), containing about 20 per cent D-glucosamine, 7-8 per cent phosphoric ester, 50 per cent long-chain fatty acid (including hydroxy-fatty acids), and a peptide side chain containing serine and dicarboxy amino acids.

Although the lipid A component of the endotoxins of the gram-negative bacteria is essential for many of the endotoxic manifestations and can act as a potent adjuvant in the production of antibodies (Westphal, 1960), the portion which determines the specificity of protective antibodies is the polysaccharide component. Such anti-polysaccharide antibodies do not protect the organism producing them against the pyrogenic effects of endotoxin if it is experimentally injected, but they do account for the species-specific immunity which generally follows recovery from an infection with one of the micro-organisms. Consequently it is the anti-polysaccharide antibodies which are of greatest interest to immunologists. Considerable progress has recently been made in the study of the chemical basis for the immunological differences which are observed, especially in the group of gram-negative bacteria known as the Salmonella. Before we can discuss them we must pause to recall a few salient facts about this important group of microorganisms.

The Salmonella

The Salmonella are Gram-negative, non-spore-forming, motile bacteria which are generally pathogenic for both man and animal. *S. typhosa,* causative agent of typhoid fever, *S. paratyphi A,* and possibly *S. sendai,* cause disease only in man.

The Salmonella are mostly flagellated. The flagella as well as the body of the organism contain antigens. The flagellar antigens are called H antigens, the somatic antigens O antigens. The letters originated with German writers who observed that colonies of the motile (i.e., flagellated) Salmonella on agar medium were surrounded by a "Hauch" (breath or emanation), while colonies of the nonmotile organisms were "Ohne Hauch" (without emanation).

The H antigens are of two kinds: those shared by a number of species or types, and those peculiar to a particular species or type, or shared by only a few species or types. Many of the species or types are *diphasic*; that is, at one stage of a culture the specific flagellar antigens may occur (specific phase), whereas at another the group antigens may be present (group phase). Any given culture of such an organism may consist entirely of one or the other of the phases or may contain both. A bacillus in one phase usually keeps

the same phase for a number of generations, but is always capable of giving rise to the other phase. As a matter of fact, the antigens of either phase may occur in various types, although the specific antigens are generally restricted to a smaller number of types (Dubos, 1945). These complicated antigenic properties of the *Salmonella* can be a source of confusion unless they are understood.

TABLE 8-2

Somatic and Flagellar Antigens in Certain Common Salmonella[a]

Group	Species	O Antigens[b]	H Antigens	
			Phase 1 (specific)	Phase 2 (group)
A	*S. paratyphosa*	(I), II, XII	a	—
B	*S. schottmuelleri*	(I), IV, (V), XII	b	1,2
	S. typhimurium	(I), IV, (V), XII	i	1,2
C_1	*S. hirschfeldii*	VI, VII,	c	1,5
	S. choleraesuis	VI, VII	c	1,5
	S. oranienburg	VI, VII	m,t	—
	S. montevideo	VI, VII	g,m,s	—
C_2	*S. newport*	VI, VIII	e,h	1,2
D	*S. typhosa*	IX, XII,	d	—
	S. enteritidis	(I), IX, XII	g,m	—
	S. gallinarum	I, IX, XII	—	—
	S. pullorum	I, IX, XII	—	—
E	*S. anatum*	III, X	e,h	1,6

[a] Modified from a table in *Bacterial and Mycotic Infections of Man*, edited by R. J. Dubos, 1952. Courtesy of Dr. Dubos, Dr. H. R. Morgan, J. B. Lippincott Co., and the National Foundation for Infantile Paralysis.

[b] Parentheses indicate that the antigen is not invariably present.

It was formerly the practice to designate the somatic (O) antigens by Roman numerals, as shown in Table 8-2, but following a decision made in 1953 at the Sixth International Congress of Bacteriology in Rome, the workers who have recently contributed so much to our knowledge of the chemical structure of these antigens, employ Arabic numerals. I shall follow this usage. It was former practice to denote the species flagellar antigens by small Roman letters, and the group flagellar antigens by Arabic numerals. Thus *Salmonella newport* possesses O antigens VI and VIII, species H antigens e and

h, and group H antigens 1 and 2. From here on, however, we shall be speaking of *S. newport* as possessing O antigens 6 and 8. The flagellar antigens will not come into the picture, because I do not intend to discuss them further.

The antigenic structure of the Salmonella has been studied in great detail by Kauffman (1937) and White (1926); the classification of these authors, based on the somatic and flagellar antigens, is in common use. In general, the species of Salmonella are divided into groups on the basis of similarity with respect to the O antigens, and the species within a group are often differentiated according to differences between their H antigens (Kauffmann, 1950, 1951). The species have been arranged in groups designated A, B, C, etc., according to similarities in the content of O antigens. All this, it should be remembered, was done purely on the basis of serological evidence.

Chemistry of the Polysaccharide Component of Salmonella Antigens

On hydrolysis, the Salmonella polysaccharides split into monosaccharides and phosphoric acid. Chromatographic study of the sugars shows that they represent a fairly complicated mixture; a single polysaccharide may consist of six to seven different sugars, including hexosamines (glucosamine and galactosamine), heptoses, hexoses, pentoses, and deoxy sugars (Davies, 1955; Mikulaszek et al., 1956; Davies, 1960). The dideoxy sugars move faster on chromatograms than the other sugars do, and their discovery, based on this property, by Staub (1952) and Westphal (1952) was a new fact of great immunochemical interest. They play a very important role in the structure of the Salmonella antigens because:

(a) Brief acid hydrolysis of the Salmonella lipoidpolysaccharides always splits off these dideoxy sugars before significant amounts of other sugars are released. This shows that the deoxy sugars are *terminal* and acid labile in the branched polysaccharide structure. It is known (see above, pp. 39-40) that the terminal groups play a predominant role in hapten specificity.

(b) When pathogenic "smooth" *Salmonella* forms change to the nonpathogenic "rough" forms, the fast chromatographic sugar components in hydrolysates of the antigens are missing, although the endotoxic lipoid A component is still present.

These dideoxy sugars are all 3,6-dideoxyhexoses, and five have so far been identified in natural antigens (Table 8-3).

TABLE 8-3

Naturally Occurring 3,6-Dideoxyhexoses[a]

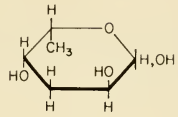
Name	First found in	References
Abequose	Endotoxin of *S. abortus equi*	Westphal, Lüderitz, Fromme, and Joseph (1953).
Tyvelose	Endotoxin of *S. typhosa*	Pon and Staub (1952), Westphal, Fromme, and Joseph (1953).
Ascarylose	Glycolipid of eggs of *Parascaris equorum*	Fouquey, Polonsky, and Lederer (1957).
Paratose	Endotoxin of *S. paratyphi*	Davies, Fromme, Lüderitz, Staub, and Westphal (1958).
Colitose	Endotoxin of *Eschenchia coli* O 111	Lüderitz, Staub, Stirm, and Westphal (1958).

[a] Westphal, 1960.

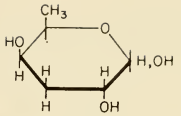
The structures of these dideoxyhexoses are shown in Fig. 8-1. It will be noted that two of them, colitose and ascarylose, have the configuration of the "unnatural" L-series of hexoses, which are suspected of playing a role in the structure of the human Rh antigens (see Chapter 7). This does not necessarily mean that any serological similarities between the Salmonella antigens and the Rh blood group antigens are to be expected, although this is a point which so far as I know has not been tested. But it does tend to confirm our suspicion that the "unnatural" sugars are more widely distributed in nature than was expected. What their relative abundance will turn out to be is another question.

Relation of Structure of Salmonella Antigens to Specificity

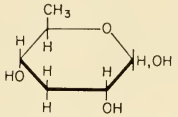
Comparison of the results of chromatographic analyses of Salmonella antigens with their position in the Kauffmann-White classification (Staub, Tinelli, Lüderitz, and Westphal, 1959; Staub, 1960; Westphal, Lüderitz, Staub, and Tinelli, 1959) showed that the



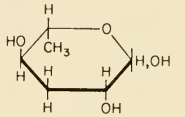
Colitose
(3,6-Dideoxy-L-galactose)



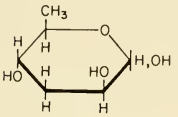
Abequose
(3,6-Dideoxy-D-galactose)



Paratose
(3,6-Dideoxy-D-glucose)



Ascarylose
(3,6-Dideoxy-L-mannose)



Tyvelose
(3,6-Dideoxy-D-mannose)

Fig. 8-1. Five naturally occurring 3,6-dideoxyhexoses.

terminal dideoxy sugars did play an important antigenic role, as expected. Each Salmonella species produces only one such sugar, and this sugar is characteristic of the group, A, B, etc., into which the species falls in the Kauffmann-White scheme. Paratose is characteristic of species in group A, for example, and colitose of group O (Table 8-4).

It has been further shown (Staub, Tinelli, Lüderitz, and Westphal, 1959) that different dideoxyhexoses function as terminal groups of various antigenic factors of the Kauffmann-White scheme, abequose being the terminal unit of antigen 4 of group B, tyvelose of antigen 9 of group D, and colitose of antigen 35 of group O.

There seems to be no evidence that more than one of these 3,6-dideoxyhexoses occurs in the endotoxin of any one species of Salmonella. When a 3,6-dideoxyhexose does occur it always occupies the terminal position in a side chain of the antigenic determinant of

TABLE 8-4

Carbohydrate Structural Units of Specific O Antigens (Endotoxins) of *Salmonella* Groups A, B, D, and O[a]
(Heptoses and aminosugars not included)

Group species	Kauffmann-White antigens	Hexoses			6-Deoxy-hexoses	3,6-Dideoxy-hexoses			
		Galactose	Glucose	Mannose	Rhamnose	Abequose	Colitose	Paratose	Tyvelose
A *S. paratyphi*	1,2,12	+	+	+	+			+	
B *S. schottmuelleri*	1,4,5,12	+	+	+	+	+			
B *S. typhimurium*	1,4,5,12	+	+	+	+	+			
B *S. abortus equi*	4,12	+	+	+	+	+			
B *S. budapest*	1,4,12	+	+	+	+	+			
B *S. stanley*	4,5,12	+	+	+	+	+			
B *S. salinatus*	4,12	+	+	+	+	+			
D *S. typhosa*	9,12	+	+	+	+				+
D *S. enteriditis*	1,9,12	+	+	+	+				+
D *S. gallinarum*	1,9,12	+	+	+	+				+
D *S. dar-es-salaam*	1,9,12	+	+	+	+				+
O *S. adelaide*	35	+	+				+		
O *S. monschaui*	35	+	+				+		

[a] Westphal, 1960.

the carbohydrate antigen. This does not mean that other sugars cannot be terminal, for glucose and rhamnose can occur in this position.

As would have been expected on the basis of what we have learned in earlier chapters of this book, the most informative way of studying the terminal sugar of these antigens was found to be by the inhibition reaction. Staub, Westphal, and colleagues (Staub and Tinelli, 1957; Staub et al., 1959) took advantage of the fact that degree of inhibition can be measured quantitatively if the reaction inhibited is the precipitation of a soluble antigen by a precipitating antibody, see above, p. 20; they made use of soluble antigens obtained by acetic acid lysis of the microorganisms and purification by Freeman's method (1942) of the product (Table 8-5).

In this table PsTy stands for the polysaccharide extracted from *S. typhosa,* PsTyB for the polysaccharide from *S. schottmuelleri* (formerly paratyphoid B), and $PsTy_{OX}$ for the carbohydrate of

TABLE 8-5

Specific Inhibition of Precipitation of Salmonella Antigens by Anti-*S. typhosa* Antiserum[a]

Inhibitor	Horse anti-typhoid serum reacted with		Rabbit anti-typhoid serum reacted with		
	PsTy[b] (12,*9*)[e]	$PsTy_{ox}$[c] (*9*)	PsTyB[d] (12)	PsTy[b] (12,*9*)	PsPtB[d] (12)
Glucose	3	2	3	58	73
Galactose	5	0	3	25	26
Mannose	4	10	0	19	25
Rhamnose	23	2	85	11	10
Tyvelose	27	66	0	7	0

[a] Staub et al., 1959. Numbers indicate per cent inhibition.

[b] Polysaccharide extracted from *S. typhosa*.

[c] PsTy oxidized with periodic acid.

[d] Polysaccharide from *S. schottmuelleri* (formerly paratyphoid B) = *S. paratyphi B*.

[e] Somatic antigens 9 and 12 of the Kauffman—White scheme. The italic number indicates the antigen which characterizes group D, the group that includes *S. typhosa*.

S. typhosa after treatment with periodic acid. The reason for including such oxidized antigens in the studies is that periodic acid destroys substances possessing two adjacent hydroxyl groups, such as terminal glucose or galactose. Terminal 3,6-dideoxyhexoses, however, do not possess such a combination of hydroxyls and are not attacked.

From the results obtained with the horse anti-typhoid serum shown in Table 8-5, Staub et al. (1959) concluded that tyvelose is the terminal sugar of antigen 9 and rhamnose that of antigen 12.

It will be seen from Table 8-5 that the results obtained with the rabbit serum were quite different from those of the horse serum. The precipitation of the polysaccharide of *S. typhosa* (PsTy) by horse anti-typhoid was inhibited significantly only by rhamnose and tyvelose, whereas these sugars inhibited precipitation of the same antigen by rabbit anti-typhoid very poorly. Glucose was much more active with rabbit serum. The difference in inhibition of precipitation of the polyoside of *S. schottmuelleri* (PsPtB) was even greater. It was therefore concluded that antigen 12, common to *S. typhosa* and

S. schottmuelleri, contains a side chain terminating in glucose as well as one terminating in rhamnose.

Similar studies carried out by Staub et al. on antisera to *S. schottmuelleri* (containing antigens 4, 5, and 12) showed that abequose inhibited the precipitation of PsPtB and especially of $PsPtB_{ox}$. This showed that abequose is the terminal unit of either antigen 4 or 5. Since abequose and antigen 4 are found in all Salmonella of group B, but antigen 5 is lacking in some members of this group, Staub et al. concluded that abequose plays no role in antigen 5. This was confirmed by the observation that the precipitation of an extract of *S. typhimurium,* which contains no 5 antigen, is inhibited by abequose and by the finding that when all the antibody precipitable by an extract of this *S. typhimurium* was removed from the anti-PsPtB serum, the action of the serum on PsPtB was not inhibited by the abequose.

Staub et al. (1959) suggest that the dideoxyhexoses may play an especially important role in the specificity of the Salmonella antigens, not only because they are always terminal, but because the two hydrophobic CH_2-groups they contain are able to approach much closer to the corresponding surface of the antibody than the hydrophilic CHOH-groups, thus strengthening the van der Waals forces between the antigenic determinants and the antibody (see Chapter 9).

Cross-Reactions

As a result of extensive studies similar to those just outlined, Staub et al. concluded that although distinct Salmonella antigens generally have different terminal sugars, this is not always the case. For instance, abequose occurs at the extremity of both antigens 4 and 8, and glucose at the extremity of antigens 1 and 12. It seems reasonable to conclude that in such cases the next-to-terminal sugar is different, or attached in a different way. In order to test this idea, the authors carried out quantitative cross-reactions with a number of polysaccharides. Some of their results are shown in Table 8-6.

From the precipitation observed with the galactomannans of gum ghatti, lucerne, and clover, Staub concluded that the Salmonella antigen 4 has structural similarities with these polysaccharides; for, whenever the antibodies to antigen 4 were removed, precipitation of

TABLE 8-6

Cross-Reactions of Horse Serums for *S. schottmuelleri* and *S. typhosa* with Certain Polysaccharides[a]

Polysaccharide	Antibody remaining for antigens	Precipitating serum absorbed with: — (*1*,5,12)	PsPtB —	$PsPtB_{ox}$ (12)	PsTy (*1*,5)	PsTm[b] (5)
1. Anti-*S. schottmuelleri* serum						
Galactomannan of						
Gum ghatti		270	19	40	—	—
Lucerne		255	28	—	243	2
Clover		200	25	—	—	—
Dextran		67	—	—	33	
2. Anti-*S. typhosa*						
	Antibody remaining for antigens	*9*,12	9			
Dextran		108	7			

[a] Staub et al., 1959. Numbers indicate micrograms of precipitate nitrogen.

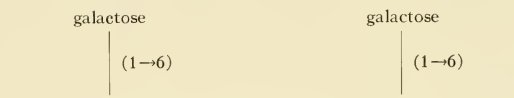
[b] Polysaccharide from *S. typhimurium*. Other antigens abbreviated as in Table 8-5.

the galactomannans was reduced virtually to zero. Antibody to antigen 12, on the other hand, seems not to cross-react with these galactomannans, as is shown by the fact that removal of anti-12 by absorption with polysaccharide of *S. typhosa* does not much affect the precipitation of the serum with lucerne.

The antibodies precipitable by dextran are seen to be, at least in part, anti-12 antibodies, since removal of anti-12 by treatment with PsTy considerably reduces the amount of precipitation with dextran. This is shown even more clearly by the fact that removal of the anti-12 from the anti-*S. typhosa* serum eliminates, for all practical purposes, precipitation with dextran.

From these results Staub and her co-workers concluded that antigen 12 contains glucose units linked as they are in dextran. They felt they could not decide whether these glucoses were in the side chain which terminates in rhamnose or part of a chain terminating

in glucose. They further concluded that antigen 4 contains groupings similar to those present in the galactomannans. These polysaccharides contain long chains of mannose linked 1→4, with occasional side chains consisting of galactose linked 1→6, as shown in the following scheme:



It is evident that the precipitability of anti-4 antibodies by these galactomannans is due to their specificity for a terminal galactose, a galactose-mannose group, or a chain of mannose linked 1→4. The last possibility is eliminated by the fact that oxidized paratyphoid polysaccharide still precipitates this antibody, for mannose linked 1→4 would be destroyed by periodic acid oxidation. The probability that the cross-reaction is due to a terminal galactose was diminished by the failure of Heidelberger and Cordoba (1956) to obtain cross-reactions with other polysaccharides containing terminal galactoses. Also, periodic acid oxidation would destroy a terminal galactose, yet the oxidized polysaccharide is still able to absorb out the anti-4 antibodies. One is, therefore, led to conclude that the grouping common to antigen 4 and the galactomannans is the galactose-mannose grouping. But, since antigen 4 terminates in a nonoxidizable sugar and the only such sugar present is abequose, the terminal portion of antigen 4 may be:

abequose—galactose—mannose

Staub et al. (1959) were able to detect a weak cross-reaction between *S. schottmuelleri* and *S. newport* owing to the terminal abequose which forms part of antigen 4 in the former and part of antigen 8 in the latter. This cross-reaction took place with horse serum only, which suggested that the horse produces antibodies specific for the terminal sugar more readily than the rabbit does.

In later work Staub et al. established the terminal sequence of sugars in antigens 1 and 12 as:

α-glucose—galactose—mannose—rhamnose

The linkages between the glucose and galactose are different in the

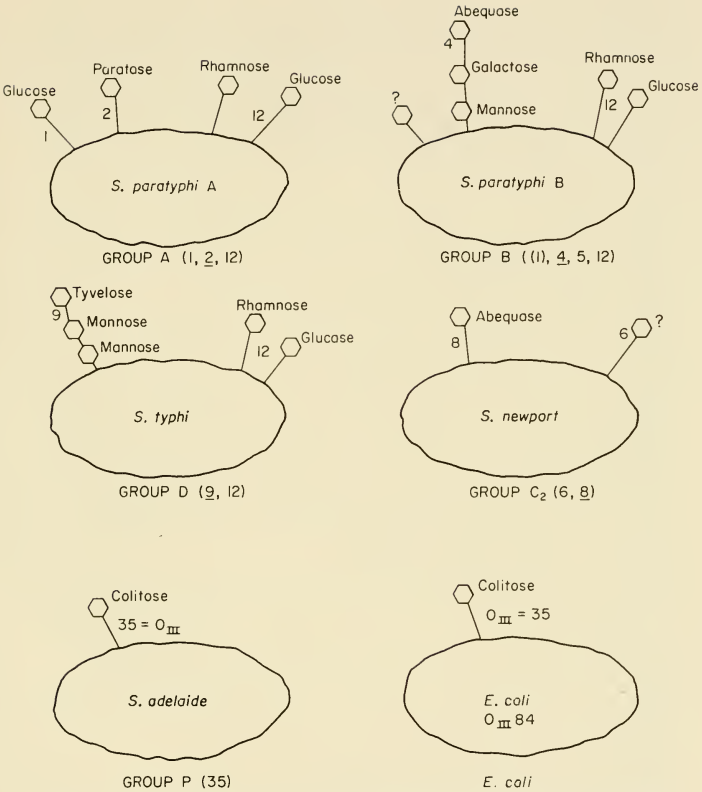


Fig. 8-2. Scheme showing our present knowledge of the role of known sugars in the specificity of some Kauffmann-White antigens (Staub, 1959). Ellipses indicate bacteria, projecting chains O antigens.

two antigens, probably 1→6 in antigen 1 and 1→4 in antigen 12. (Staub, 1960; Stocker, Staub, Tinelli, and Kopacka, 1960; Tinelli and Staub, 1960).

A summary of the conclusions of Westphal, Staub, et al. about the

antigenic structure of several Salmonella species in terms of chemical structure of the Kauffmann-White classification is shown in Fig. 8-2. To anybody familiar with the (largely unavoidable) vagueness of serological methods of classifying bacteria, the concreteness of the new results will seem like a ray of light in a dark room. We may confidently anticipate that this ray will grow brighter until the whole intricate structure is illuminated.

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CHAPTER 9

Union of Antibody with Antigen: Thermodynamics

The exact mechanisms by which antibodies produce their effects have not been cleared up in all cases, but that the first step is combination of the antibody and antigen is not in dispute. It is therefore of interest to inquire into the forces involved and the firmness of the union. A proper treatment of these points will require the introduction of a few elementary thermodynamic notions.

Forces Involved

Landsteiner (1936) pointed out that the covalent bond (e.g., the bond holding the two carbons together in ethane, $H_3C—CH_3$) does not generally form fast enough and is not reversible enough to be a plausible explanation of antibody-antigen reaction and that some compounds which can react with antibodies cannot form covalent bonds. Similar arguments probably rule out the coordinate link or semipolar double bond.

We are left with three possibilities: coulomb forces, van der Waals forces, and hydrogen bonding. Coulomb forces are those causing positive and negative charges to attract each other. All antibodies and many antigens are proteins, and it is pertinent to remark that prominent among the charged groups in protein molecules are the positive free ϵ-amino groups $—NH_3^+$ of lysine and the negatively charged free carboxy groups $—COO^-$ of the dicarboxylic amino acids such as aspartic acid. A separated, fixed, pair of positive and negative charges constitutes a dipole. It is easy to see how dipoles

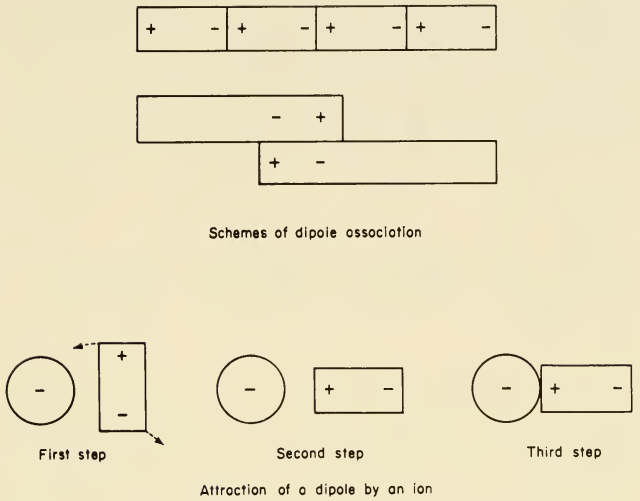


Fig. 9-1. Schemes showing dipole-dipole association and attraction of a dipole by an ion.

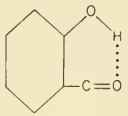
may attract other dipoles as a result of coulomb forces, or attract positive or negative ions (Fig. 9-1).

Van der Waals forces constitute the most general intermolecular attraction and may operate between any two molecules. They depend not upon permanent but upon instantaneous dipole moments. A molecule which has no permanent dipole moment, for example methane (CH_4), may have at a certain instant an instantaneous dipole moment when the center of charge of the rapidly moving negative electrons surrounding the carbon nucleus lies to one side of the center of charge of the positive nucleus. This instantaneous dipole moment produces an instantaneous electric field which may influence another molecule in the immediate neighborhood. As a result the electrons of the second molecule move relative to their nucleus in such a way as to produce a force of attraction for the first molecule.

Van der Waals forces decrease very rapidly with distance, being inversely proportional to the seventh power of the distance, and are

consequently negligible when two molecules are separated by any appreciable distance. They are quite strong between molecules that can bring parts of their "surfaces" into close contact.

Hydrogen bonds (also relatively short-range) consist essentially of a hydrogen atom which is attracted simultaneously to two different atoms. For example, the two oxygens in salicylaldehyde (Fig. 9-2)



Salicylaldehyde

Fig. 9-2.

are connected by a hydrogen bond. Many of the unusual properties of water are due to hydrogen bonding. It is believed that hydrogen bonds play an important part in maintaining the characteristic configurations of protein molecules.

The role of coulomb forces in holding antibody and antigen together was removed from the realm of pure hypothesis by the experiments of Singer (1957). This worker pointed out that if a negatively charged group is involved in an antibody-antigen bond, it is possible to calculate the effect of pH on antibody-antigen combination. The assumption is made that, if the negative group is in the antigen, the antibody contains a corresponding positively charged group, and vice versa. For our present purposes it is immaterial which molecule contains the negative group. Singer and Campbell (Singer, 1957) suggested that, if there is one negative group characterized by an intrinsic hydrogen ion association constant K_H and if we neglect the nonspecific repulsion between antibody (Ab) and antigen (AG) molecules, the following relation should hold in the acid region:

$$\log\ (1/K - 1/K_0)\ = \log(K_H/K_0) - \text{pH} \qquad (1)$$

where K is the apparent equilibrium constant at a given pH for the reaction

$$\text{Ab} + \text{Ag} \rightleftarrows \text{AbAg}$$

and K_0 is the value of K at neutral pH where both the positive and

the negative group are fully ionized. A similar relation would apply in the alkaline region. If two negative and two positive groups were critically involved in each Ab-Ag bond, the expected relation would now contain a $(pH)^2$ and a $2(pH)$ term.

Singer tested this relation by ultracentrifugal observations on Ab-Ag mixtures at different pH. Typical results are shown in Fig. 9-3,

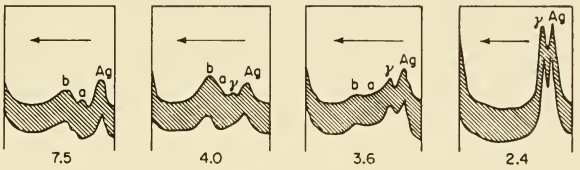


Fig. 9-3. Ultracentrifugal diagrams of mixtures of bovine serum albumin and its antibody at various pH. Sedimentation is proceeding in the direction of the arrow. Ag stands for antigen, Ab for antibody, *a* for an antibody-antigen complex thought to be Ag_2Ab, *b* represents a complex thought to be Ag_3Ab_2, and δ is gamma globulin. At pH less than 4.5, progressively larger amounts of free gamma globulin (antibody) appear, while the amounts of the complexes diminish (Singer, 1957).

which shows the sedimentation diagrams of mixtures of bovine serum albumin and rabbit anti-bovine serum albumin. As the pH falls, more and more free gamma globulin (antibody) appears in the mixture while the amount of the antibody-antigen complexes decreases. The changes are clearly a function of pH* and were found to be entirely reversible.

Enough results at different pH were obtained to show that the linear relation predicted by the equation holds quite well (Fig. 9-4). This was found to be true for both systems studied, namely, ovalbumin-antiovalbumin and bovine serum albumin-antibovine-serum-

* Habeeb et al. (1959), however, conclude from chemical modification studies that "the removal of the positive charge on the same amino groups of Ab by an increase of the pH of the solution, instead of by acetylation, might have the same effect on the Ab molecule and its capacity to precipitate with a large Ag molecule. The generally-observed dissociation of Ag-Ab bonds in alkaline solution might therefore be attributable to such a deformation of the Ab molecule, rather than . . . to titration of specific critical groups within the Ab sites."

albumin. The constant K_{II} had in both cases a value of about 10^5, which is consistent with the idea that a carboxyl group, —COO^-, is critically involved in the antibody-antigen bond in these systems, and must be ionized for maximum bond strength. Singer concluded that the attraction of this group for its complementary positive group accounts for about half of the strength of the antibody-antigen bond in these cases. The remainder is presumably due to some or all of the other forces mentioned above.

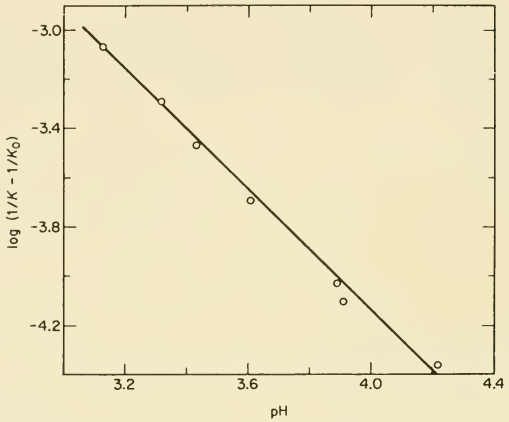


Fig. 9-4. Effect of pH on antibody-antigen equilibrium in the bovine serum albumin system, plotted according to equation (1). The slope of the line is —1.2 (Singer, 1957).

In the case of the attraction of antibody to *p*-(*p*′azophenylazo)-benzene arsonate, Nisonoff and Pressman (1957) found that the negatively charged —COO^- group contributed over 4.8 kcal./mole to the binding energy, again indicating the presence of a positive charge in the combining group of the antibody. The uncharged *p*-phenylazo group contributed 2.3 kcal./mole.

There are a number of reasons for believing that the van der Waals forces are among the most important of the non-coulomb forces. One of the arguments supporting this belief derives from the fact that the

strength of the antibody-antigen bond is greatly decreased if the hapten or antigen combining group is slightly changed in shape. This is shown by work with haptens of known chemical constitution, such as the experiments discussed in Chapter 1, and by measurements of the bond strength for groups of related haptens, discussed below. The strong influence of shape suggests that close contact between the various atoms of the combining group of the antibody and the atoms of the haptens or antigenic combining group is necessary for a strong antibody-antigen bond. Such closeness of contact accords well with the suggestion, made by Hooker and Boyd (1941), Pauling and Pressman (1945) (Fig. 2-12), and Karush (1956), that the combining group of the antibody may in fact be a cavity into which the hapten or antigen combining group fits snugly. Close fit would make the van der Waals forces strong, and any change in the hapten or antigen combining group that lessened that fit would markedly weaken the strength of the bond, which is precisely what we observe.

Although in the two systems studied by Singer the non-coulomb forces (which, if the argument in the preceding paragraph is valid, may be second in importance) were thought to account for only about half the strength of the antibody-antigen bond, there are cases where the non-coulomb forces presumably account for the entire bond strength. These cases apply to antigens which do not contain positively or negatively charged groups in their specifically reactive portions. Good examples of such antigens are provided by the blood group substances (Chapter 7). Here, no positive or negative groups are present, at least not in the portions responsible for the antigenic specificity. Yet the blood group antigens combine firmly and typically not only with antibody but with the blood group-specific plant proteins I have called lectins (Chapter 6). These reactions have been studied quantitatively (Kabat, 1956; Boyd, Shapleigh, and McMaster, 1955). Karush (1958) believes that the forces between antibody and carbohydrate antigens are mainly hydrogen bonds.

Wurmser and Filitti-Wurmser (1950) suggest that the combining energy of the isohemagglutinins with their receptors on the human erythrocyte is equivalent to that of about four hydrogen bonds or twenty van der Waals bonds. Before we can discuss such quantitative estimates further it will be necessary to go into some of the concepts of thermodynamics.

Energy

We shall need to discuss only the first two laws of thermodynamics. The first law is well known and today needs only to be stated to be believed. It is simply that energy can neither be created nor be destroyed. It is understood that we are not thinking of changes involving changes in atomic nuclei; if we were, we should have to formulate the law more broadly.

From the first law of thermodynamics it follows that no perpetual motion machine of the "first type," i.e., one getting all or part of its energy from nowhere, can ever be constructed. The total energy of a completely isolated system, therefore, remains constant (If the system is not isolated its total energy may change from time to time.) We designate this total energy, which may be made up of heat (which Count Rumford proved to be a form of energy) or of mechanical or chemical energy and at times of other forms, as E. The science of thermodynamics grew out of a study of the process by which heat may be converted by suitable machines partly into work. If we let Q stand for the heat content of the system and W for the work *done,* we may write the simple equation

$$\Delta E = \Delta Q - \Delta W \tag{2}$$

which states that the increase in the total energy of the system, ΔE, equals the heat taken up, ΔQ, minus the work done, ΔW. This is a statement of the first law of thermodynamics in symbols.

If we consider an extremely small change in the system and ignore certain questions of mathematical rigor, we may replace the finite differences ΔE, ΔQ, and ΔW by the differentials dE, dQ, and dW, and write

$$dE = dQ - dW \tag{3}$$

The meaning of this equation is not as obvious as the beginner might think. It looks as if the equation means that, if you measure the infinitesimal increase in the total energy of a system, you can show experimentally that it equals the experimentally determined infinitesimal absorption of heat minus the experimentally determined amount of work done. But this is not the meaning at all, for we have no "energy meter" with which we can measure the total energy of a system, or even the change in total energy. The only way we have

of getting dE is by measuring dQ and dW and taking the difference. It looks as if equation (3) is a trivial tautology.

This is not the case, however, because there is an essential difference between dE on one hand and dQ and dW on the other (Klotz, 1950). For dE is an exact differential, and dQ and dW are not. The meaning of the mathematical term exact differential is discussed in textbooks of the calculus. Here we need only recall that, if a differential dX is exact, the values of X at two different points, X_1 and X_2, depend solely on the initial and final values of the independent variables of which X is a function, whereas, if dX is inexact, the values of X depend upon the particular route we take from X_1 to X_2. In physics, if the pressure P and volume V of steam in an engine are fixed, the values of the other variables such as the temperature T are thereby determined. Since the values of P and V determine the state of the system, P and V are called the independent variables. We could have chosen other sets of two, such as P and T or V and T, but in the study of heat engines, where thermodynamics originated, the set P, V is particularly useful.

We find that specifying P and V does not uniquely determine Q or W, for the amount of heat a system may take up can vary in spite of this, and it is well known that the portion of the heat a machine converts into work depends on the efficiency of the machine. Consequently, dQ and dW are inexact differentials and final values of Q and W depend not merely on the final values of P and V, but on the route we choose in getting from the state P_1, V_1 to P_2, V_2. Two possible routes are shown schematically in Fig. 9-5.

On the other hand, the value of E is completely determined by P and V, and no matter what route we take from P_1, V_1 to P_2, V_2, the final value of E, E_2, will be the same. Consequently, if we go from point 1 to point 2, then back to point 1 (this we call a reversible cyclic process), ΔE must equal zero, while ΔQ and ΔW will in general be different from zero. All this is a mere restatement of the first law of thermodynamics, but it is of the greatest importance.

A thermodynamic quantity which depends only upon the values of the independent variables is called a *thermodynamic function*. Thus, the total energy E is such a function. Knowing that E is a thermodynamic function, we can write other expressions which are also thermodynamic functions. For example, if we write

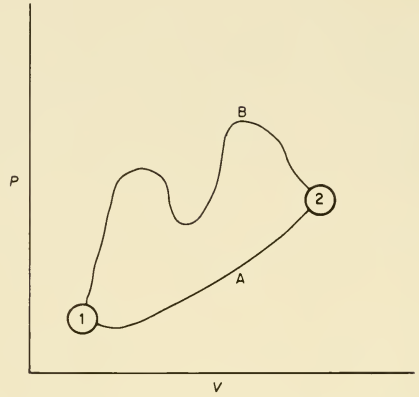


Fig. 9-5. Two possible reversible routes from state 1 to state 2.

$$H = E + PV \tag{4}$$

it is obvious that H is a thermodynamic function, for we have seen that E depends solely on the values of P and V, and the product PV depends only on these variables. Therefore dH is an exact differential. The quantity H is called the total heat, or enthalpy. Enthalpy is important because, when the pressure remains constant, which it does during most chemical reactions, the change in enthalpy is equal to the heat absorbed or heat given off, as follows:

$$\pm(\Delta H)_P = \pm(\Delta Q)_P \tag{5}$$

The subscript P indicates that the variable P (pressure) remains constant. The sign convention, positive for heat absorbed and negative for heat given off, was more natural in the study of heat engines than it is in chemistry, but is now firmly established.

ΔH is interesting because in many cases a large negative ΔH for a chemical reaction goes along with a strong tendency for the reaction to go spontaneously. Indeed it was long believed that $-(\Delta H)_P$ was the proper measure of the spontaneity of a reaction. It was eventually found that not $-(\Delta H)_P$ but the change in another thermodynamic function, the free energy, is the proper measure of the

spontaneity of a chemical reaction.* The more spontaneous a reaction, the stronger the chemical bonds formed. But before we can discuss free energy we must introduce the second law of thermodynamics.

Entropy

Going back to equation (3), we may rewrite it as follows:

$$dQ = dE + dW \tag{6}$$

If the pressure on a system remains constant, any work done is the product of the change in volume times the pressure, thus

$$dQ = dE + P\,dV \tag{7}$$

Since we know that E is a function of P and V, we have, by an elementary and purely formal application of the calculus

$$dE = (\partial E/\partial V)\,dV + (\partial E/\partial P)\,dP \tag{8}$$

where ∂ indicates partial differentiation. Substituting in equation (6), we obtain

$$dQ = (\partial E/\partial V + P)\,dV + (\partial E/\partial P)\,dP \tag{9}$$

Since dQ is not an exact differential, equation (9) cannot be integrated as it stands. It is shown in the calculus (e.g., Osgood 1925) that, whenever you have an equation of the form

$$dQ = X\,dV + Y\,dP \tag{10}$$

where X and Y are functions of the variables P and V, there is always an integrating factor $B = f(P,V)$, in fact a number of such

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*A reaction may be spontaneous and, nevertheless, not take place at any appreciable rate of speed under ordinary conditions. For example, the reaction

$$2H_2 + O_2 \rightarrow 2H_2O$$

has a large negative ΔH and is also spontaneous by the free energy criterion (see below). Nevertheless, mixtures of gaseous hydrogen and oxygen can be stored indefinitely at ordinary temperatures and pressures. The reaction is spontaneous, however, as is clear from what happens when an electric spark is passed through the mixture.

functions of P and V, such that when both sides of equation (10) are multiplied by one of them, the product $B\,dQ$ becomes an exact differential. In the present case such a function is easily found. The simplest one is $1/T$. Multiplying by $1/T$, we obtain

$$dQ/T = [(\partial E/\partial V + P)/T]\,dV + [(\partial E/\partial P)/T]\,dP \qquad (11)$$

That dQ/T is an exact differential is proved in thermodynamics (Klotz, 1950) by showing that dQ/T is an exact differential (a) for an ideal gas carried through a certain sequence of reversible changes called a Carnot cycle, (b) for any substance carried through a Carnot cycle, and (c) for any substance carried through any reversible cycle.

The sequence of changes which constitute a Carnot cycle is so simple and symmetrical that it is easy to show that, for such a cycle and by virtue of part (c) of the above-mentioned proof for any reversible cycle,

$$W/Q_2 = (T_2 - T_1)/T_2 \qquad (12)$$

where W is the work done by the system during the cycle, Q_2 is the heat taken in at the higher temperature T_2, and T_1 is the lower temperature. The fraction W/Q_2 is called the efficiency of the cycle. In thermodynamics it is further proved that (a) the efficiency of a real substance carried through a Carnot cycle cannot be greater than that of an ideal gas and cannot be less, and (b) the efficiency of any substance carried through any reversible cycle is the same as that of an ideal gas carried through a Carnot cycle. The fraction W/Q_2 is therefore the maximum theoretical efficiency of any heat engine which takes in heat Q_2 at temperature T_2 and returns part of the heat to the surroundings at temperature T_1. The efficiency of an actual engine will be less than this; it is impossible for the efficiency of any engine, actual or theoretical, to be more.

Since dQ/T is an exact differential, it can be integrated. As a result of this integration we shall obtain a function of the independent variables P and V. This is a new thermodynamic function, and we can give it a name. The name of the new function is *entropy*. It is represented by the symbol S, and we write

$$dS = dQ/T \qquad (13)$$

The discovery that the integral of dQ/T* defines a new thermodynamic function constitutes also a discovery of the second law of thermodynamics. It is probably the best way of introducing the concept of entropy, which is not, like the concepts of temperature, pressure, heat content, etc., an obvious generalization of ideas already more or less familiar to the non-scientist but a subtle and powerful new concept. The best attempt to explain the concept in words is to say that it is a measure of the *disorder* of a system, or of the extent of the loss of availability of energy.

The second law of thermodynamics can be stated in words in a number of other ways, though none of them adequately suggests the significance and applicability of the principle. For example, we may say that no heat engine can produce work by taking a quantity of heat from the environment at a certain temperature and returning the unused heat to the environment at the same temperature. Such an engine would be a perpetual motion machine of the "second type," and the second law of thermodynamics asserts that no such machine can ever be constructed.

The significant thing about the second law for chemists is that it provides a valid measure of the tendency of a process to take place. when the change in entropy

$$\Delta S_{V,E}$$

(the subscripts meaning volume and energy are constant) is large and positive, the process will tend to take place spontaneously, and this tendency is greater the larger $\Delta S_{V,E}$.

Free Energy

Although fine for the processes that take place in heat engines, as a measure of the spontaneity of a chemical reaction $\Delta S_{V,E}$ has its drawbacks. In chemical reactions, the volume of the system often does not remain the same and the energy practically never does. Pressure and temperature are usually constant but volume and entropy vary. Consequently, we want a new thermodynamic function,

* The dQ in the definition of entropy must be the heat absorbed in a reversible process and is sometimes written explicitly dQ_{rev}.

let us say F, such that $F_{T,P} = f(V,S)$. We can get this rather simply by the definition

$$F = H - TS \tag{14}$$

It is easy to show that F is a function of V and S when P and T are constant. So is TS, obviously: when T is constant it is a function of S alone. From our original definition of H we have

$$H = E + PV \tag{15}$$

When P is constant, PV is a function of V only. We saw above that E is a function of P and V only; consequently, when P is constant, E is a function merely of V. Therefore,

$$F = H - TS = E + PV - TS \tag{16}$$

is a function of V and S. Consequently, the thermodynamic function F defined by this expression is a function of V and S. The new function is called the Gibbs free energy.

When T and P are constant, we have from equation (16)

$$\Delta E_{P,T} = \Delta E + P\,\Delta V - T\,\Delta S \tag{17}$$

Now, from equation (3) above, we have $\Delta E = \Delta Q - \Delta W$. If we ignore complications such as osmotic effects, the only work the system does is mechanical, $\Delta W = P\,\Delta V$, and $\Delta E = \Delta Q - P\,\Delta V$. Substituting this into equation (17), we obtain

$$\Delta F_{P,T} = \Delta Q - P\,\Delta V + P\,\Delta V - T\,\Delta S \tag{18}$$

From the definition of entropy, $\Delta Q = T\,\Delta S$ for a reversible process, we find that for a reversible process, or at equilibrium,

$$\Delta F_{P,T} = 0 \tag{19}$$

If the pressure does vary but the temperature continues to remain constant, we have from equation (16)

$$dF = dE + P\,dV + V\,dP - T\,dS$$

Again, $dE = dQ - P\,dV = T\,dS - P\,dV$, and we obtain

$$dF_T = V dP$$

For a perfect gas we have $PV = nRT$, or $V = nRT/P$, so that

$$dF = -(nRT\,dP)/P$$

Integrating, we obtain

$$F_1 - F_2 = -\Delta F = nRT \ln(P_1/P_2) \qquad (20)$$

Spontaneously, a perfect gas can only expand; it cannot spontaneously contract. In other words, the pressure can only decrease. From this we see that in a spontaneous reaction ΔF will be negative. The larger the negative value of ΔF, the greater the tendency of the process to go.

Strictly, equation (20) applies only to a perfect gas. But it also applies without serious error to many real gases. If we replace P_2 and P_1 by the thermodynamic activities, which for the dilute solutions used in immunochemistry do not differ appreciably from the molar concentrations, we may apply this equation to antibody and antigen solutions.

Free Energy and Equilibrium

We now proceed to derive an important relation between ΔF and the equilibrium constant of a chemical reaction. Let us suppose we have a reaction between two perfect gases A and B, to give two other perfect gases, C and D. Then if we represent the numbers of moles involved by lower case letters, a, b, c, and d, the initial pressures as P_A and P_B, and the final pressures as P_C and P_D, we have to write

$$aA(P_A) + bB(P_B) \rightarrow cC(P_C) + dD(P_D) + \Delta F \qquad (21)$$

where ΔF represents the change in free energy which accompanies the reaction. In order to compare free energy changes, and therefore tendencies of reactions to take place, we need free energy changes where the starting and stopping points are always the same; in other words, all reactants must start at a standard state and finish up in a standard state. In the case of gases the standard state is atmospheric pressure. In the case of dissolved substances, which we mostly deal with in immunochemistry, the standard state is unit activity.

We can find the free energy change, called the standard free energy change and represented by ΔF°, which would result if the

reacting gases shown in equation (21) started at atmospheric pressure and the products ended up at atmospheric pressure. We simply systematically add to equation (21) a series of equations, each one of which carries one of the gases from the standard pressure to the partial pressure P_A, P_B, etc., actually observed, adding also each time the free energy change which such a change in pressure entails. For instance, the first equation we add is

$$aA(P_A = 1) \rightarrow aA(P_A = P_A), \quad \Delta F = aRT \ln(P_A/1) \qquad (22)$$

After performing all these additions we combine the logarithmic terms and obtain

$$\Delta F^\circ = \Delta F + RT \ln[(P_A)^a(P_B)^b/(P_C)^c(P_D)^d]$$

or

$$\Delta F^\circ = \Delta F - RT \ln[(P_C)^c(P_D)^d/(P_A)^a(P_B)^b] \qquad (23)$$

If the amounts a, b, etc., and the pressures P_A, P_B, etc., are those found at equilibrium, the free energy change ΔF in the reaction shown in equation (21) is zero, and the term ΔF drops out. And since we see that the expression whose natural logarithm appears in (23) is in that case simply the equilibrium constant K, equation (23) reduces to

$$\Delta F^\circ = -RT \ln K \qquad (24)$$

which is the relation we were seeking. Again we see that when there is a strong tendency for the reaction as written to go to the right (K is large), ΔF° will be large and negative.

The equilibrium constant of a reaction is a measure of the extent to which a reaction goes to completion. The standard free energy change, which can be calculated from it, is thus a proper measure of the strength of the chemical bonds that are formed, and broken, during the reaction. Whenever the equilibrium constant of a reaction can be measured, we can calculate the standard free energy change. If we know ΔF°, we can calculate the entropy change ΔS°, if ΔH° is known from calorimetric measurements, by using equation (14) in the form $\Delta F^\circ = \Delta H^\circ - T\,\Delta S^\circ$. ΔH° has been measured directly for only a few immunochemical reactions. When it cannot be measured it can often be calculated from van't Hoff's equation

$$d(\ln K)/dT = \Delta H^\circ/RT^2 \tag{25}$$

If we assume ΔH° is independent of T in the range of temperatures studied, we can integrate equation (25) to obtain

$$\ln(K_2/K_1) = -(\Delta H^\circ/R)[1/T_1 + 1/T_2] \tag{26}$$

which makes it possible to estimate ΔH° if observations on the equilibrium constant are available at two different temperatures. In fact this is the commonest way of obtaining ΔH°.

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CHAPTER 10

Energy of Antibody-Antigen Reactions

Direct Calorimetry

In the early days of immunochemistry, methods were not available for measuring the amounts of free antigen or antibody, or both, remaining after antibody and antigen have reacted. Therefore calculation of the free energy change from direct measurements of the equilibrium constant was not possible. The earlier estimates of the strength of the antibody-antigen bond were based on attempts to measure the heat of reaction ΔH. It will be seen from equation (14) in the previous chapter, which we can rewrite as follows,

$$\Delta F^\circ = \Delta H^\circ - T\Delta S^\circ \quad T = \text{const.} \tag{1}$$

that if the entropy change were zero, ΔH° would equal ΔF°, and such a measurement would be an adequate measure of the strength of the antibody-antigen bond. In fact, we may regard equation (14) as a statement that in order to make ΔH° a reliable index of the tendency of the reaction to take place, we have to correct it by allowing for the entropy change ΔS°. A positive entropy change will make a negative ΔF° still more negative, a negative entropy change will make it less negative or even positive. Somewhat unexpectedly, recent work suggests that in serological reactions ΔS° is, in fact, not large, though usually not zero.

Nevertheless, not too much has been learned about antibody-antigen reactions by direct calorimetry. The first attempt, by Bayne-Jones (1925), gave results that we now know were nearly a million times the correct value. Two later determinations, the first by

Kistiakowsky and his group (Boyd et al., 1941), and the second by Steiner and Kitzinger (1956), gave −40 and −6 kcal per mole of antibody, respectively. I doubt if this difference was due to experimental error; more likely it should be traced to differences in features between the two very different antibody-antigen systems used. Steiner and Kitzinger's result agrees better with values of ΔH° calculated indirectly for other serological reactions, as we shall see below.

Free Energy from Equilibrium Measurements

Various methods have been used to measure the equilibrium between free antibody and antigen and their compounds, or between antibody and hapten and their compounds, including (i) equilibrium dialysis, (ii) direct analyses of precipitates and supernatants, (iii) electrophoretic and ultracentrifugal observations, and (iv) light scattering. Details of the experimental procedures will have to be found in the references cited. Here we may say merely that all are methods of determining or calculating the concentrations at equilibrium of free antibody, free antigen, free rapten, or compounds thereof. From such measurements the equilibrium constant K and ΔF° can be calculated. If measurements can be made at more than one temperature, ΔH° and ΔS° can also be estimated.

Of the above methods, (i) and (iv) are applicable only to simple antibody-hapten systems, the former only to univalent hapten systems. Method (iii) can be applied to systems in which the antibody is reacting with a protein, but application of the method may in some cases disturb somewhat the very equilibrium which it is desired to measure. Method (iv) does not disturb the equilibrium.

In applying method (iii), allowance must be made for the fact that antibody is divalent, at least usually, and protein antigens are multivalent (Epstein, Doty, and Boyd, 1956). Therefore, if we measure the equilibrium in which each antibody is combined with as many antigen molecules as possible (two in the case of divalent antibody), in the presence of free antigen and the compound AG, where A represents antibody and G represents antigen, our equilibrium constant corresponds to

$$(AG_2)/(G)\ (AG) = K \qquad (2)$$

What we are interested in, however, is the strength of a single antibody-antigen bond. The compound AG_2 contains two such bonds, and each mole of free antigen G contains v' moles of free combining sites, where v' is the valence of the antigen. Consequently, we have to obtain the value of K', where K' is the equilibrium constant corresponding to the equilibrium

$$\frac{\text{(antibody-antigen bonds)}}{\text{(free antigen sites) (free antibody sites)}} = K' \quad (3)$$

by writing

$$2(AG_2)/v(G)\ (AG) = K'$$

or

$$K' = (2/v)K \quad (4)$$

Therefore, the standard free energy of a single antibody-antigen bond ΔF°_i equals $-RT \ln K' = -RT \ln K - RT \ln(2/v)$, or

$$\Delta F^\circ_i = -RT \ln K + RT \ln(v/2) \quad (5)$$

The exact value of the correction will depend on the valence of the antigen and the exact nature of the reaction the equilibrium state of which is being studied.

As an illustration, let us consider the results of Baker et al. (1956) on the reaction of anti-benzenearsonic acid antibodies with benzenearsonic acid-azo-bovine serum albumin (bovine serum albumin coupled with diazotized arsanilic acid). The reaction studied by these workers was

$$AG + AG \rightleftarrows AG_2$$

and their bovine serum albumin contained thirteen introduced benzenearsonic acid azo groups per molecule. They calculated a ΔF° of -5.2 kcal. per mole. From the above this is equivalent to a bond free energy change ΔF_i° of

$$-5.2 + RT \ln(13/2)$$

or

$$-5.2 + 1.1 = -4.1 \text{ kcal./bond}$$

Contrary to expectations, this value is less (i.e., more positive) than

the value of —7.4 kcal. per bond found by Epstein, Doty, and Boyd (1956) for the reaction of anti-benzenearsonic acid antibodies with the divalent hapten T (terephthalanilide-*p,p'*-diarsonic acid) (Fig. 10-1).

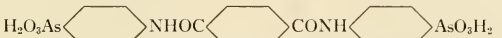

Fig. 10-1. Divalent hapten used by Epstein, Doty, and Boyd (1956).

It would have been expected that the benzenearsonic acid groups in the coupled bovine serum albumin, being coupled through the azo linkage with tyrosine and histidine residues just as in the coupled protein used for immunization, would correspond to the combining sites of the antibody better than the amide-coupled benzenearsonic acid groups of the hapten T. Epstein, Doty, and Boyd suggested that the decreased bond strength was due to some unfavorable feature in the orientation of the groups in the coupled protein.

In dealing with multivalent antigens which may combine simultaneously with a number of molecules of antibody, the mathematical problems of formulating the reaction become formidable unless we introduce simplifying assumptions. The simplest assumption is that the free energy of combination of an antibody molecule with a combining site of the antigen is the same for all such sites and is not affected by the number of antibody molecules which have already combined with the antigen. With the aid of this assumption, which can hardly be strictly true but which is certainly adequate as a first approximation, we can easily solve the problem, as shown by Linderstrøm-Lang (1924), von Muralt (1930), Fowler (1936), Wyman (1943), and Klotz (1946). If we let the association constant for the formation of a single antibody-antigen bond be K, and the number of combining sites on the antigen molecule (or cell) be m, we find the ratio r of antibody molecules combined with an antigen molecule (or cell) to be

$$r = mK(\mathrm{A})/[1 + K(\mathrm{A})] \tag{6}$$

where (A) is the concentration of free antibody.

A summary of the principal thermodynamic studies on the antibody-antigen or antibody-hapten reaction is given in Table 10-1.

TABLE 10-1

Thermodynamic Values Reported for Serological Reactions

Antigen or hapten[a]	Reaction studied[b]	Methods[c]	Reference	$\Delta F°$, kcal./mole	$\Delta H°$, kcal./mole	$\Delta S°$, e.u.
1. Hcy	A	DM	(2)	-10[d]	-40	-100
2. HSA	C	DM + (iii) + (iv)	(12)	$-7.5 - 8.0$	-3.66	$13.1 - 14.8$
3. BSA	D	(iii)	(9)	-5.5 ± 0.2	0 ± 2	20 ± 8
4. O	D	(iii)	(10)	-5.6 ± 0.2	0 ± 2	20 ± 8
5. Carboxy peptidase	B	(ii)	(11)	-9	-7	7
6. R-BSG	B	(ii)	(5)	*ca.* -8.5	-2	21
7. R-BSA	D	(i)	(1)	-5.2 ± 0.2	0 ± 2	18 ± 8
8. S-BSA	B	(ii)	(5)	-8.5	-2.8	21
9. Hapten T	F	(iv)	(4)	-7.4 ± 0.2	-0.8 ± 2.6	22 ± 9
10. DNP	E	(i)	(3)	-6.8	-1.6	17
11. D-I:	E	(i)	(7)	-7.24	-7.1	0.3
12. Lac	E	(i)	(8)	-7.25	-9.7	-8.8
C. Dye B		(i)	(6)	-6.54	-3.93	8.75

[a] Hcy = hemocyanin, HSA = human serum albumin, BSA = bovine serum albumin, BSG = bovine serum globulin, DNP = ϵ-dinitro-phenyllysine, O = avalbumin. Structures of R, S, and other haptenic groups shown in Fig. 10-2. The antibody used in study 1 was horse antibody, rabbit antibody was used in other studies. Study C was on the binding of benzoic acid dye (B, Fig. 10-2) by normal BSA.

[b] See Table 10-1a.

[c] DM = direct measurement; other methods as described in text, p. 135.

[d] Assumed value.

TABLE 10-1a

Reactions for which Data Are Presented in Table 10-1

A. $nA + G \rightleftarrows A_nG$
B. $A + A_{n-1}G \rightleftarrows A_nG$
C. $A + 2G \rightleftarrows AG_2$
D. $AG + G \rightleftarrows AG_2$
E. $A + 2H \rightleftarrows AH_2$
F. Haptenic group + antibody site $\rightleftarrows$ hapten-antibody bond

A = antibody, G = antigen, H = hapten.

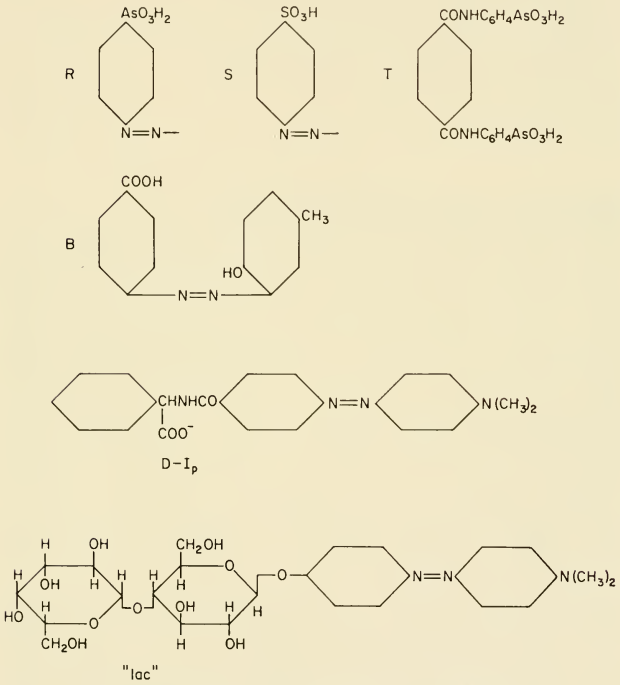

Fig. 10-2. Structures of haptens referred to in Table 10-1.

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Significance of Thermodynamic Constants

The figures in Table 10-1 present some unexpected features. Most surprising, perhaps, is that ΔF° is generally not large; —9 kcal per mole seems to be about an upper limit. This is not a large value for standard free energy changes. The free energy of formation of water, for example, is —54.65 kcal. per mole (for two hydrogen-oxygen bonds); that of carbon monoxide is —33.0 kcal. per mole (for one carbon-oxygen bond). On the other hand, it can be seen from Fig. 10-3 that the free energy changes involved in the formation of the antibody-antigen bond are sufficient to cause the reaction to go substantially to completion if the reagents are concentrated. (This figure shows the relation between the equilibrium constant K and the free energy change. Also shown is the per cent of product B at equilibrium in a hypothetical reaction $A \rightleftharpoons B$.)

Not only are the values of ΔF° small by physical and chemical standards, but the values for the different reactions are surprisingly alike, suggesting that no antibody-antigen reaction is likely to have a large free energy change. If antibody is formed through contact with a molecule or portion of a molecule of antigen or with some

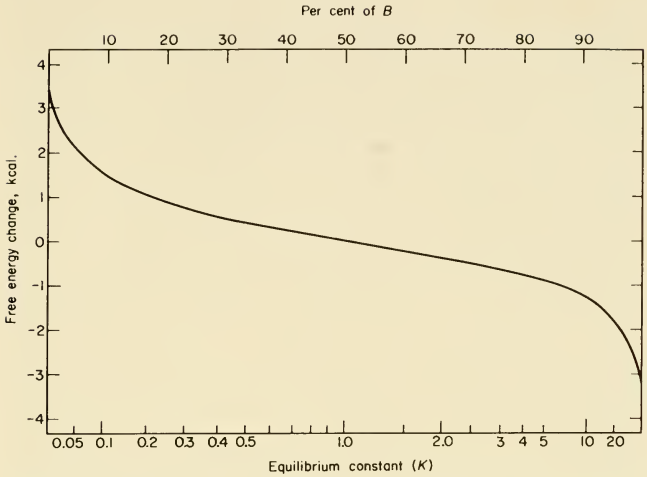


Fig. 10-3. Relation between standard free energy change (ΔF°) of a reaction $A \rightleftarrows B$ and the equilibrium constant. Also shown is relation between ΔF° and the per cent composition of the equilibrium mixture with respect to B. (Slightly modified from H. B. Bull, 1951, *Physical Biochemistry,* 2nd ed., Wiley, New York, by permission).

intracellular template which causes part of the new molecule to have a configuration complementary to the antigenic determinant, a small value for ΔF° is understandable. An antibody molecule that possessed too strong an affinity for the fixed antigen molecule or intracellular template would have difficulty leaving its place of formation and getting into the circulation, as has been pointed out by Pauling (1940) and Singer (1957).

Another unexpected feature of Table 10-1 is that the values of ΔS°, with two exceptions, are positive instead of negative. When antibody molecules combine with a molecule of antigen, their freedom of motion is restricted, and this loss of freedom constitutes a loss of "configurational entropy." Therefore, one would expect antibody-antigen reactions to be accompanied by a decrease in entropy. The positive values reported therefore demand explanation.

It has generally been supposed that the positive values for ΔS°

are due to the fact that in most cases there is mutual neutralization of positive and negative charges (see p. 120), with resulting loss of attraction for water molecules. Restoring freedom of motion to water molecules previously bound to the antigen or antibody surface causes an increase in entropy, and this might be more than enough to compensate for the loss of entropy due to the decreased mobility of the antibody molecules. For instance, Epstein, Doty, and Boyd (1956) calculated that in the reaction studied by them the release of about twenty-four water molecules accounted for the observed $\Delta S°$. In line with this argument, Karush (1958) found a negative entropy change of about nine units for the reaction of antibody with his lactose-hapten "lac," where there is no charge to be neutralized.

The one large negative entropy change in study 1 of Table 10-1 is harder to explain. However, it should be remembered that, in the first place, it is based on a value of $\Delta F°$ which was merely assumed and, in the second place, hemocyanin is a rather special antigen in a number of ways, being much larger and more multivalent than most antigens and constituting an associating and dissociating system. Steiner and Kitzinger (1956) suggested that a change in the state of association of the hemocyanin might account for the large enthalpy change observed and for the large negative entropy change calculated from this value.

A third feature of the results of Table 10-1 is that the enthalpy (heat content) changes are small, with the exception, again, of that found in study 1. Aside from this perhaps atypical value, the largest enthalpy change in the table is the —9.7 kcal. per mole calculated by Karush (1958) for the reaction of antibody with the "lac" hapten. This is definitely on the small side when compared with the $\Delta H°$ of —94.03 kcal. per mole for the reaction of hydrogen and oxygen to form water, or the —26.4 kcal. per mole for the reaction of carbon and oxygen to form carbon monoxide. It is also of interest that, in all cases where $\Delta H°$ is not zero, or so close to zero that its exact magnitude is not known, it is negative. i.e., the reaction is exothermic.

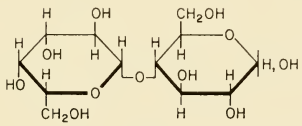
The enthalpy changes of all the antibody-antigen or antibody-hapten reactions studied, with the exception of that in study 1, are too small to account for the firmness of the bond and the fact that the reaction goes to substantial completion. Obviously, in many, perhaps most, cases the major portion of the driving force of the reaction $\Delta F°$ is

contributed by the term $T\,\Delta S^\circ$ and is thus due to the positive entropy change (equation (25).

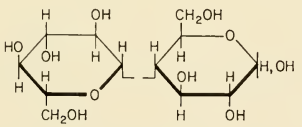
In spite of the relative weakness of the antibody-antigen or antibody-hapten bond, antibodies display very sharp specificity, as we have already seen. For instance, when Karush compared the reactions of anti-"lac" antibody with lactose with the reaction of the same antibody with cellobiose, he found a value for ΔF° of -5.52 kcal. per mole for lactose and only -1.96 kcal. per mole for celloboise, although the only difference between the two sugars is the arrangement of the hydrogen and hydroxyl groups on carbon number 4 of the terminal hexose unit (Fig. 10-4). This again accords with the notion that the hapten fits quite precisely into a portion of the antibody.

The importance of close fit of antibody to hapten is also shown by the work of Nisonoff and Pressman (1957) who found that substitution of an iodine atom ortho to the carboxy group of the benzoate ion decreased the antibody-hapten combining energy by 2.4 kcal. per mole. Substitution of an iodine in the meta position decreased the binding energy by about 0.7 kcal. per mole.

It has been known for some time that the antibody molecules in any given antiserum are heterogenous (references in Boyd 1956). This heterogeneity manifests itself, among other ways, by differences in their specific affinity (Karush, 1958; Epstein, Doty, and Boyd, 1956; Nisonoff and Pressman, 1958). This means that the ΔF°



Lactose



Cellobiose

Fig. 10-4.

for the antibodies of an antiserum will be different for the different antibodies; combination of antigen or hapten will be firmer with some than with others. Heterogeneity of antibody is responsible, for example, for the fact that the relation between r/c and r, where r is the average number of hapten molecules bound per antibody molecule and c is the concentration of free hapten, is not a linear one (see Fig. 10-5). It was suggested by Pauling, Pressman, and Grossberg (1944) that the standard free energy of combination of the various antibody molecules may follow the distribution of the normal error function (Gaussian distribution) (see Fig. 2-4). This suggestion has been worked out in detail by Karush (Karush and Sonenberg, 1949; Karush, 1956).

If K_0 is the average binding constant and σ a measure of the

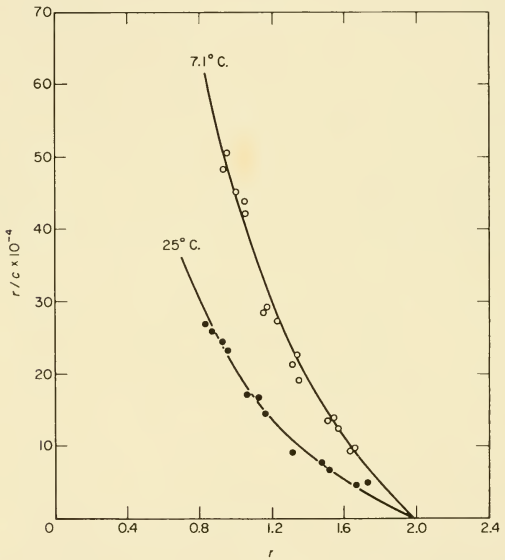


Fig. 10-5. Binding results at 25°C. and 7.1°C, for the reaction between D-I_p hapten and purified anti-D-I_p antibody (Karush, 1957, 1958). The points are experimental and the curves theoretical.

range of values of K, then the "normalized" Gaussian function (Pauling, Campbell, and Grossberg, 1944) is

$$[1/\sqrt{(\pi)}\sigma] \exp[- \ln(K/K_0)/\sigma]^2$$

The fraction of total combining sites, n, which have a specified binding constant K will be, for an infinitesimally small area dn, expressed as follows:

$$dn/n = [1/\sqrt{(\pi)}\sigma] \exp[- \ln(K/K_0/\sigma]^2 \, d \ln(K/K_0)$$

From this Karush and Sonenberg (1949) found (the derivation is given by Klotz, 1953) that the fraction of antibody sites occupied, r/n, where n is the number of combining sites per antibody (found by Karush to be two in confirmation of much earlier work), is in terms of the concentration c of free hapten, as follows:

$$r/n = 1 - [1/\sqrt{(\pi)}] \int_{-\infty}^{\infty} \{[1 - \exp(-\alpha^2)]/[1 + K_0 c \exp(\alpha\sigma)]\} \, d\alpha$$

where α is $[\ln(K/K_0)]/\sigma$. Karush (1957, 1958) found that if for his D-I_p anti-D-I_p system he took the heterogeneity index σ of antibody to be 2.3, the above equation enabled him to account satisfactorily for the experimentally formed relation between r/c and r. (See Fig. 10-5, where the circles are the experimental points and the curves are theoretical.)

Heat of Reaction of Isoagglutinins

The thermodynamic constants for the reaction of the human isohemagglutinins have been estimated by Wurmser and Filitti-Wurmser (Filitti-Wurmser, Jacquot-Armand, and Aubel-Lesure, and Wurmser, 1954; Wurmser and Filitti-Wurmser, 1957), who have devoted a great deal of penetrating thought and experimental skill to the problem. The methods used are somewhat different from those involved in the studies just discussed and deserve a little space to themselves.

Wurmser and co-workers showed that the combination of isoagglutinins with human erythrocytes is reversible, so that equilibrium considerations apply. We can use equation (25), which gives us a relation between the equilibrium constant K and the concentration of free antibody at equilibrium. Equation (6) contains two unknown constants: the number of combining sites on a red cell, m, and the

association constant K. Precise values of m are not yet available, but Wurmser and Filitti-Wurmser devised methods of calculation which did not require a knowledge of m.

If we invert both sides of equation (6), we obtain

$$1/r = 1/m + 1/mK(\mathrm{A}) \tag{7}$$

This means that if we plot the reciprocal of the number of moles of agglutinin combined with a mole of red cells against the reciprocal of the concentration of free agglutinin, we should get a straight line with slope $1/mK$. If we make such determinations at two different temperatures, the ratio of the two slopes $(1/mK_2)/(1/mK_1)$ gives us the ratio of the association constants at these two temperatures, K_1/K_2. From this we may calculate ΔH° from van't Hoff's equation (25) (p. 133).

The amount of isoagglutinin remaining free in equilibrated mixtures of erythrocytes and serum cannot be estimated with sufficient accuracy by the method of serial dilutions generally used to estimate the strength of an agglutinating serum, and the quantitative methods of Heidelberger and his school are not sensitive enough. But the Wurmsers hit upon the device of expressing the agglutinin content of their sera in terms of the maximum number of red cells they agglutinate, and of determining the free agglutinin in the supernatant of erythrocyte-agglutinin mixtures in the same way. This enabled them merely by cell counting to obtain the data for determining the requisite slopes and ratios of slopes described in the last paragraph (Fig. 10-6).

The values of ΔH° calculated by these methods are shown in Table 10-2. It will be seen that these values of ΔH° are in several cases larger than the rather small values calculated by other workers for other antibody-antigen and antibody-hapten systems. The most surprising feature of Table 10-2, however, is the marked differences in the anti-B isoagglutinin values obtained from the blood of persons of different blood group and even of different genotype. This has been confirmed by the examination of the serum of 36 A_1O individuals, six of group A_1A_1, and eight of group OO. The anti-B in the serum of any given individual seems always to be homogeneous. This homogeneity is in marked contrast to the heterogeneity found for immune antibodies (p. 14) and, if confirmed, might go far toward supporting

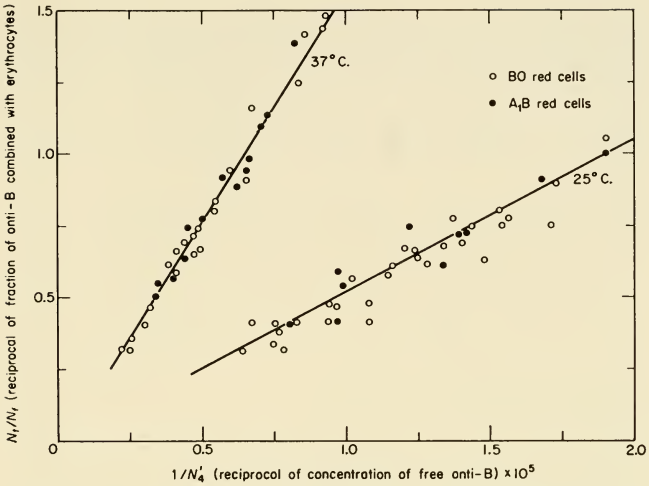


Fig. 10-6. Relation between reciprocals of fraction of anti-B agglutinin combined with erythrocytes and concentration of free anti-B agglutinin, at 37°C. and 25°C., showing linear relationship and different slopes at the two temperatures (Filitti-Wurmser et al., 1954).

TABLE 10-2

Heat of Combination of Isohemagglutinins with Erythrocytes[a]

Isoagglutinin	Agglutinogen	Genotype of donor	$\Delta H°$,kcal./mole
Anti-B	B	A_1O	-16 ± 2
Anti-B	B	A_1A_1	-6.5 ± 1.1
Anti-B	B	A_2O	-9
Anti-B	B	OO	-1.7 ± 0.4
Anti-A	A_1	BO	-10 ± 3
Anti-A_1	A_1	BO	-33 ± 2.5

[a] Wurmser and Filitti-Wurmser, 1957.

the views of workers such as Furuhata (1927) who postulated that the isoagglutinins anti-A and anti-B were as much a product of the blood group genes as the agglutinogens A and B were.*

*On the whole the less probable view, see Chapter 4, p. 57.

By estimating the molecular weights of the isoagglutinins and by estimating m by determining the amount of protein nitrogen taken up by erythrocytes from agglutinating sera, Wurmser and Filitti-Wurmser were able also to obtain approximate values for the free energy and entropy changes for these anti-B-B reactions. The approximate molecular weights obtained are shown in Table 10-3.

TABLE 10-3

Approximate Molecular Weights of Human Anti-B Isoagglutinins[a]

Genotype of donor	M[b]
A_1O	~500,000
A_1A_1	~200,000
OO	~125,000

[a] Wurmser and Filitti-Wurmser, 1957.
[b] Molecular weight.

It will be seen that the different kinds of anti-B, according to Wurmser, also differ in molecular weight.

The calculated free energy and entropy changes are shown in Table 10-4. It will be seen that these results suggest that the binding energies ΔF° are not very different for the three kinds of anti-B, but that the differences in ΔH° correspond to significant differences in ΔS°. Wurmser and Filitti-Wurmser concluded that the specific combining groups of the three different kinds of anti-B are not very different and suggested that the increase in entropy which results when the anti-B of group O serum combines with B erythrocytes may be connected with a perturbation of the entire protein molecule, pos-

TABLE 10-4

Free Energy and Entropy Changes for Binding of Anti-B Isoagglutinins by B Erythrocytes[a]

Genotype of donor	ΔF°,kcal./mole	ΔS°,e.u.
OO	−9.2	+24
A_1A_1	−9.5	+9.7
A_1O	−9.8	−20

[a] Wurmser and Filitti-Wurmser, 1957.

sibly some sort of reversible denaturation which results in greater disorder and consequent absorption of heat. These effects might mask the evolution of heat and the decrease in entropy which are caused by the local reaction of the specific combining group with the B receptor on the cell.

According to Wurmser and Filitti-Wurmser, these findings indicate that the isoagglutinins in man are produced not by a process of immunization, as antibodies are in general, but directly under control of the blood group genes. This view, if correct, would sharply distinguish these "natural agglutinins" from those produced by immunizing animals, or presumably even from those produced by injection of A and B blood group substances into human volunteers.

Kabat (1956) does not believe such a distinction exists, and does not believe there are such substances as "natural agglutinins." He has also criticized the calculations of Wurmser and Filitti-Wurmser in detail, but to me none of his criticisms seem conclusive; indeed, some seem quite beside the point. (Cf. Filitti-Wurmser, Jacquot-Armand, and Wurmser, 1960.) A decision as to the validity of the Wurmser and Filitti-Wurmser conclusions will have to await confirmation or disproof of their work in another laboratory.

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